UNIVERSITY OF LONDON THESIS

Degree Ph.D. Year 2006 Name of Author MANFREDI, M.

Characteristics of *Candida* isolates from patients with diabetes mellitus

Thesis submitted by

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-2006-

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Abstract

The present research has investigated the molecular characterization of oral yeasts in healthy individuals from different dental clinical settings in the United Kingdom, and patients from the UK and Italy who were affected by diabetes mellitus (DM) to determine the impact of DM upon candidal infection of the mouth.

In the present study of patients with DM from the UK and Italy a lower than expected incidence of oral candidal infections (7/249, 2.8%) was observed. The presence of oral yeasts and genotypic diversity of *C. albicans* was also not influenced by DM. There was no significant difference between the presence of oral yeasts in Italian and UK DM patients. However, more ($p=0.04$) *C. dubliniensis* isolates were found in non-DM individuals.

Higher levels of *Candida* adhesion to fibronectin-coated paramagnetic beads were observed in isolates from DM patients. Isolates from patients with low oral yeast loads adhered significantly ($p=0.01$) more than those from patients with high oral yeast loads. In general, there was no difference in proteolytic activity of isolates from DM or control patients.

Candida isolates from UK DM patients had significantly greater *in vitro* resistance to azole antifungal agents (miconazole $p<0.0001$; fluconazole $p=0.02$; ketoconazole $p=0.01$) than those from Italian DM patients. In addition, *C. albicans* isolates from all examined patients were more susceptible to fluconazole ($p=0.0008$) and miconazole ($p=0.01$) than non-*C. albicans* strains.

PCR fingerprinting and subsequent phylogenetic analysis revealed that *C. albicans* isolates from UK DM patients were the most diverse ($p<0.0001$) in comparison to those from Italian DM patients or from healthy subjects.

Finally, it was observed that the activity of a rat IgM monoclonal anti-idiotypic antibody (mAbK10) and a synthetic decapeptide (KP) had a significant dose-

dependant fungicidal activity upon a wide spectrum of *C. albicans* and non-*C. albicans* isolates from patients with and without DM.

Declaration

I hereby declare that this thesis is the result of my own work, unless otherwise stated. It contains neither material previously published or written by another author, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at the University or other institute of higher learning, except where acknowledgments have been made in the text.

Maddalena Manfredi
London, 06-02-2006

Publications as a result of this thesis

Papers

1. Al-Kaarawi ZM, Manfredi M, Waugh ACW, Jorge J, McCullough MJ, Scully C, Porter SR. Molecular characterisation of *Candida* spp. isolated from the oral cavities of patients from diverse clinical settings.
Oral Microbiol Immunol 2002; 17:44-49.

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Acknowledgements

I am indebted to my supervisor Professor S.R. Porter for his valuable guidance and support during the course of my PhD. I am also grateful to my second supervisor Dr. M.J. McCullough for his input, knowledge and help with the thesis, particularly regarding Chapter 7, PCR fingerprinting.

A particular thanks goes to Professor Luciano Polonelli and Professor Stefania Conti for their invaluable support, ideas and for the excellent training facilities provided for me at the University of Parma in the latter part of my PhD. They gave me the opportunity to work with KP and K10 and test these two new molecules on my *Candida* isolates.

I am also indebted to Professor Giuseppe Pedrazzi who kindly helped me to review the statistical analysis of this thesis.

I would like to express my gratitude to Professor PierUmberto Gennari, who was the first person to give me the opportunity to dedicate my time to this research degree and to Professors Mauro Bonanini and Silvia Pizzi who have constantly supported my research.

I would particularly like to thank Professor Paolo Vescovi for his support and guidance over the years.

A thank you is also due to all my colleagues in the Oral Medicine Department at Eastman Dental Institute and in the Sezione di Microbiologia (Microbiology Department) for their encouragement and assistance in the laboratory-based studies, particularly to Zubaida Al-Kaarawi, Alastair Waugh, Jacks Jorge, Antonella Salati, Lara Ravanetti, Simona Arseni and Raffaele Frazzi. I would also like to thank the clinical staff at Middlesex Hospital, the University College of London and the Endocrinology Department at the University of Parma.

I would also like to thank my dear friends and colleagues: Dr Mario Fazzi, Dr Elisabetta Merigo, Dr Marco Meleti and Dr Alessandra Dei Cas who have always found the time to help me.

I will always be grateful for the friendship and support of Cristina Bez and Liz Hill for always keeping my spirits and hopes above water and for being there when I needed them.

Finally, I would like to thank my parents and my brother, Michele, without whom this thesis would never have come into being and Andrea for his love and support throughout my PhD.

Abbreviations

ABC: ATP-binding cassette genes

AIDS: acquired immune deficiency syndrome

ALS: agglutinin-like sequence genes

AP-PCR: arbitrarily primed polymerase chain reaction

ATCC: American-type culture collection

ATP: adenosine triphosphate

BEC: buccal epithelial cells

bp: base pair

BSA: bovine serum albumin

Candida spp.: *Candida* species

c-DNA: complementary DNA

CDR: complementary-determining regions

CES: candidosis endocrinopathy syndrome

cfu: colony forming units

CHC: chronic hyperplastic candidosis

CMC: chronic mucocutaneous candidosis

DM: diabetes mellitus

DNA: deoxyribonucleic acid

dNTP: deoxynuclotide triphosphate

dsRNA: double-stranded RNA

ECM: extracellular matrix

EDTA: ethlene-diamine-tetraacetate

EFG: elongation factor gene

ELISA: enzyme linked immunosorbent assay

FDA: Food and Drug Association

5-FC: 5-fluorocytosine

5-FU: 5-fluorouracil

h: hour(s)

$HbA1_c$: glycosylated haemoglobin

HeLa: Henrietta Lacks cells (human epithelial cells from a fatal cervical carcinoma infected by Human Papilloma Virus 18 - HPV18)

HIV: human immunodeficiency virus

IdAb: anti-idiotypic antibody

Ig: immunoglobulin

Ig A: immunoglobulin class A

IgG: immunoglobulin class G

IR-PCR: inter repeat PCR

ITS: intergenic transcribed spacer regions

KP: killer peptide, derivated from P6 with alanine scanning procedure

KT-IdAb: KT-like anti-idiotypic antibodies

KTmAb-K10: KT-like anti-idiotypic antibodies produced in monoclonal format

KTscFv-H6 (scFvH6): KT-like anti-idiotypic antibodies produced in recombinant single-chain fragment variable format

KT(s): killer toxin(s)

KTR: killer toxin receptor

LB: Lennox L broth base

LIP: lipase genes

mAb: monoclonal antibody

mAbKT4: KT-neutralizing monoclonal antibody

MF: major facilitator genes

min: minute(s)

MTL: mating-type-locus

NCCLS: National Committee for Clinical Laboratory Standards

OD: optical density

PaKT: *Pichia anomala* killer toxin

PAS: periodic acid Shiff

PAUP: phylogenetic analysis using parsimony

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PL: phospholipase genes

PLMN: polymorphonuclear leukocytes

P6: synthetic decapeptide pertaining to CDR-L1 with a remarkable candidacidal activity *in vitro*

RAPD: random amplified polymorphic DNA

rDNA: ribosomal RNA

REA: restriction enzyme analysis

RFLPs: restriction fragment length polymorphisms

RGD: arginine-glycine-aspartic acid

RNA: ribonucleic acid

RT-PCR: reverse transcriptase polymerase chain reaction

s: second(s)

SAP: secreted aspartic proteinase genes

Sap: secreted aspartic proteinases

scFv: single-chain fragment variable

SDA: Sabouraud's dextrose agar

SD: standard deviation

SDS: sodium dodecyl sulphate

sIgA: salivary immunoglobulin class A

TAE: tris acetate EDTA

TE: tris EDTA

TCA: trichloracetic acid

UPGMA: unweighted pair group method with arithmetic mean

UV: ultraviolet

VL: variable light chain

WO-1: white-opaque phase

YPD: yeast peptone dextrose

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CHAPTER 1

Introduction

1.1 *Candida* epidemiological aspects

The genus *Candida* is a collection of approximately 150 asporogenus yeast species. It is well established that only a small number of these are human pathogens, since approximately 65% of *Candida* species are unable to grow at a temperature of 37°C (Schauer & Hanschke, 1999).

Although a sexual stage (teleomorph) for *C. albicans* has historically not been detected (thus this species has been classified in the class of *Fungi imperfecti*) (Kwon-Chung & Bennet, 1992), a mating-type-like (MTL) locus has recently been identified in *C. albicans*, which is involved in meiotic differentiation in *S. cerevisiae*, suggesting the existence of a sexual cycle for this diploid fungus (Hull *et al*, 2000; Magee & Magee, 2000; Tzung *et al*, 2001). For some of the *Candida* species for which a teleomorph stage has been described, such as *C. krusei*, *C. guilliermondii*, *C. lusitaniae*, the sexual stages are ascomycetous (Kurtzman & Fell, 1998; Calderone, 2002). Furthermore, comparative sequence data from different studies supports the idea that both sexual and non-sexual *Candida* spp. are related phlyogenetically to the *Ascomycete* class of fungi (Calderone, 2002).

The presence of *Candida* by itself is not indicative of disease. *Candida* species are ubiquitous organisms and most of them are commensals, or at least transient commensals in the gastrointestinal tract (Samaranayake, 1990). *Candida albicans*, the most common human commensal of the genus *Candida*, is generally regarded as the most virulent of the *Candida* species being recovered from 70 to 80% of individuals. However, more recently (during the 1980s and 1990s), epidemiological data reveals a

mycological shift from *C. albicans* to non-*C. albicans* species, such as *C. glabrata, C. tropicalis, C. parapsilosis, C. krusei* and *C. lusitaniae* (Fidel *et al*, 1999; Moran *et al*, 2002). In 1995, a novel *Candida* species, *C. dubliniensis*, was identified for the first time (Sullivan *et al*, 1995) in Dublin, Ireland. Oral carriage and infection with this species was prevalent in HIV (human immunodeficiency virus)-infected and AIDS (acquired immune deficiency syndrome) patients. This changing pattern of *Candida* carriage and the increased incidence of *Candida* infections is a result of several different factors, such as the emergence of HIV-diseases, the increased use of immunosuppressive therapies and the widespread use of broad-spectrum antibiotics (Calderone, 2002).

In addition, the transfer of *Candida* between individuals often occurs via the hands of health care workers (Strausbaugh *et al*, 1994) and nosocomial transmission can also occur (Schmid *et al*, 1995; Cannon & Chaffin, 1999). Furthermore, it has been reported that treatment with azole antifungals for protracted periods may lead to the selection of non-*C. albicans* species, some of which have been shown to be less susceptible to these agents than *C. albicans* (Bodey, 1986; Chavanet *et al*, 1994; Ruhnke *et al*, 1994; Tumbarello *et al*, 1996; Schoofs *et al*, 1998; Fidel *et al*, 1999; Moran *et al*, 2002).

It can be difficult to determine the precise frequency of oral carriage of *Candida* species (Odds, 1988b; MacFarlane, 1990; Cannon & Chaffin, 1999; Ruhnke, 2002) as this is influenced by population age, the health of the sample population, the presence or absence of oral prostheses and the techniques used for oral sampling, yeast isolation and identification. It is estimated that about 40% of healthy individuals, without signs of oral candidosis, harbour *Candida* in their mouth as a commensal. The rate of carriage of *Candida* in saliva increases with pregnancy, tobacco smoking and when dentures are worn (Ellepola & Samaranayake, 2000a). Several studies have

reported that oral *Candida* carriage is higher in hospitalised patients (54.7%), but high counts of *Candida* species do not necessarily mean that patients will have clinical signs and symptoms of oral candidosis (Scully *et al*, 1994; Al Karaawi *et al*, 2002). Although *C. albicans* is still the most frequently recovered yeast (70-75%) from the oral cavity, several studies have indicated higher incidences of the non-*C. albicans* species (7%) such as *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis* and *C. dubliniensis.* These species are often recovered from a mixed population and usually in combination with *C. albicans* (Odds, 1988b; Sullivan & Coleman, 1998; Cannon & Chaffin, 1999).

It has become possible to understand the epidemiology of *Candida* infection and colonisation more clearly due to the introduction of reliable identification and typing systems to evaluate strain homology. Such techniques are able to make a distinction between different *Candida* species and strains that share phenotypical traits. In the past, this inability to differentiate between species and strains may have led to an underestimation of the prevalence of colonisation and infection of non-*C. albicans* spp.

1.1.1 *Candida albicans*

As previously reported, *C. albicans* is the most common *Candida* species isolated from the oral cavity of healthy subjects or from patients affected by different diseases (Cannon & Chaffin, 1999; Leung *et al*, 2000). *Candida albicans* is usually characterised by the production *in vitro* of true hyphae as detected by the germ tube test (Section 1.6.2.2) although this is a feature also evident with *C. dubliniensis* (Section 1.1.2). Over the years, a variety of studies have investigated the pathogenic attributes of *C. albicans* and these have recently been reviewed by a variety of authors (Cannon & Chaffin, 1999; Hoyer, 2001; Calderone & Gow, 2002; Chauhan *et*

al, 2002; Douglas, 2003; Naglik *et al*, 2003). In comparison with *C. glabrata* or *C. krusei* (Sections 1.1.3-1.1.4), *C. albicans* is usually more sensitive to conventional antifungal agents (Section 1.4, Table 1.3), but some resistance has been reported, particularly to azole therapy in severely immunodepressed patients (Yoon *et al*, 1999; Rex *et al*, 2000).

1.1.2 *Candida dubliniensis*

Candida dubliniensis was first isolated in 1995 from the oral cavities of HIV-infected individuals, with and without acquired immune deficiency syndrome (AIDS). Since this time, the species has been widely detected from a variety of sources including the oral cavities of DM patients (Willis *et al*, 1999; Willis *et al*, 2000b; Manfredi *et al*, 2002). *Candida dubliniensis* is rarely isolated from healthy individuals (Coleman *et al*, 1997; Sullivan & Coleman, 1997; Sullivan & Coleman, 1998). It is usually present in a mixed culture with other *Candida* species, particularly *C. albicans*. However, pure cultures of *C. dubliniensis* have been isolated from HIV-infected patients with clinical signs of oral candidosis (Coleman *et al*, 1997). The most common clinical manifestation of oral candidosis in this group of patients and caused by *C. dubliniensis* is erythematous candidosis (Table 1.2) (Sullivan & Coleman, 1998). *Candida dubliniensis* has also been isolated from faecal, sputum, vaginal and blood cultures (Odds *et al*, 1998; Meis *et al*, 2000; Polacheck *et al*, 2000; Moran *et al*, 2002). Numerous studies have reported the mis-identification of *C. dubliniensis* as *C. albicans* but it is now considered that this species can emerge as an opportunistic pathogen in severe immunodeficiency from the commensal microbial flora (Moran *et al*, 2002). Although *C. dubliniensis* is not inherently less susceptible to antifungal drugs (Pfaller *et al*, 1999b; Moran *et al*, 2002), susceptible isolates can readily

develop resistance to fluconazole *in vitro* when exposed to this agent (Moran *et al*, 1998).

1.1.3 *Candida glabrata*

As with *C. dubliniensis*, it is now evident that *C. glabrata* can cause oral infection (Fidel *et al*, 1999). There are now reports of increased *C. glabrata* infection in immunocompromised and hospitalised patients, indeed *C. glabrata* is the most commonly recovered non-*C. albicans* species from the oral cavity of HIV-infected patients (Occhipinti *et al*, 1994; Pfaller *et al*, 1999b). Nosocomial acquisition of *C. glabrata* is not uncommon. Furthermore, two major risk factors associated with *C. glabrata* colonisation are prolonged duration of hospitalisation and prior antimicrobial use (Fidel *et al*, 1999). Of clinical concern, several studies have noted that a significant proportion of *C. glabrata* isolates are resistant to fluconazole (9%) and itraconazole (37-40%) (Price *et al*, 1994; Rex *et al*, 1997; Pfaller *et al*, 1998a; Barchiesi *et al*, 1999; Moran *et al*, 2002). Fluconazole resistance can also develop rapidly in a clinical environment (Moran *et al*, 2002).

1.1.4 *Candida krusei*

Candida krusei can replace *C. albicans* in the oral cavities of HIV-infected patients, particularly after azole therapy (Chavanet *et al*, 1994; Lischewski *et al*, 1995; Coleman *et al*, 1997; Ruhnke *et al*, 2000). It is now generally accepted that *C. krusei* is inherently resistant to fluconazole, and that fluconazole prophylaxis may promote the proliferation of this organism (Rex *et al*, 2000; Moran *et al*, 2002).

1.1.5 Other important *Candida* species

Other pathogenic species of *Candida* such as *C. parapsilosis* and *C. tropicalis* are generally recovered from blood cultures and the skin of immunocompromised patients, particularly in hospital environments, but these species are rarely isolated from the oral cavity (Levin *et al*, 1998; De Bernardis *et al*, 1999b; Moran *et al*, 2002). *Candida lusitaniae* is a rare pathogen, mainly isolated from immunocompromised patients where it is often responsible for candidemia (Pfaller, 1996). *Candida lusitaniae* can easily develop resistance to amphotericin B (Moran *et al*, 2002). Like all pathogenic microorganisms, *Candida* species have developed different virulence mechanisms that confer the ability to colonize a host surface, to penetrate into deeper host tissue, and to evade host defences (Hube & Naglik, 2002) that are described in Section 1.2.

1.2 Virulence attributes of *Candida* spp.

In order to promote successful colonization or invasion of host tissues, *Candida* spp. have developed different virulence attributes. The most investigated factors are related to the *Candida* cell wall, adherence to tissues and surfaces and production of proteinases, which are all discussed in the following sections.

1.2.1 *Candida* cell wall

Of all the *Candida* spp., the composition of the cell wall has been mostly studied in *C. albicans* through different approaches, mainly based on biochemical analysis. *Candida albicans* has a cell wall that is primarily composed of glucans (40-60%), chitin (25-6%) and mannan (23%); however, lipids and proteins are also present (Calderone & Braun, 1991). The cell wall is considered essential for *C. albicans* pathogenicity, not only because it provides sites for adhesion to host surfaces, but it

acts as a barrier against osmotic pressure and environmental insult (McCullough *et al*, 1996). There is comprehensive literature on this subject, which focuses on the most important properties of this essential fungal structure (Calderone & Braun, 1991; Chaffin *et al*, 1998; Cannon & Chaffin, 1999; Chauhan *et al*, 2002).

1.2.2 Adhesion

Adhesion is a necessary step for *Candida* spp. to colonize and invade a host eco-system. The oral cavity presents many niches for *Candida* colonisation, and yeasts are able to adhere to many ligands such as epithelial and bacterial cell surface molecules, extracellular matrix (ECM) proteins and dental acrylics. Salivary molecules, including basic proline-rich proteins, adsorbed to many oral surfaces, also promote *Candida* adherence (Cannon & Chaffin, 1999; Cotter & Kavanagh, 2000). Adherence to host tissue is the first step in the pathogenic process of *Candida* infections: once first contact with the host surface has been made, enzymes facilitate adherence by damaging or degrading host cell membranes and extracellular proteins (Cotter & Kavanagh, 2000).

The mechanisms of adherence of *Candida* species to many cell types or surfaces are complex and aspects of adhesion are unclear, although it is known that adherence is achieved through a combination of specific (ligand receptor interaction) and non-specific mechanisms (electrostatic forces, aggregation, cell surface hydrophobicity) (Cotter & Kavanagh, 2000). Specific adherence is mediated by a number of target proteins located on the endothelial cell or within the ECM. With respect to the latter, fibronectins are adhesive glycoproteins located in the ECM interstitium, which have a high molecular weight and are involved in cell adhesion and cell migration (Castellani *et al*, 1986; Cotter & Kavanagh, 2000). Other microorganisms apart from *Candida*, such as *Staphylococcus aureus* and *Streptococcus pyogenes*, have also been

shown *in vitro* to adhere to the ECM protein, fibronectin (Skerl *et al*, 1984). Trans-membrane integrins present on the surface of *C. albicans* are thought to mediate adherence to ECM (Hostetter, 1994) by recognising ligands that contain amino acid sequences such as the Arginine-Glycine-Aspartic acid (RGD) sequence (Varner & Cheresh, 1996). *Candida albicans* also binds to RGD sequence-containing proteins, such as fibronectin, laminin and collagen types I and IV, through integrin-related structures (Klotz *et al*, 1994). Adherence of *Candida* to epithelial cells is also mediated by cell-wall mannoproteins, particularly the protein portion (Cotter & Kavanagh, 2000), as pre-treatment with a variety of proteolytic enzymes inhibits adherence significantly (Douglas, 1986).

Interestingly, most of the genes that encode proteins whose expression correlates with hyphal formation, also encode cell-wall mannoproteins (Calderone & Gow, 2002). Whilst nine ALS (agglutinin-like sequence) genes that encode cell-surface glycoproteins had been originally detected in *C. albicans* (Hoyer & Hecht, 2000; Hoyer, 2001; Hoyer & Hecht, 2001), the homologous nature of two of these (ALS3-ALS8) has been recently shown (Zhao *et al*, 2004): these proteins are thought to play roles in adhesion with the host.

A novel mechanism that *C. albicans* hyphae use to adhere specifically to buccal epithelial cells (BECs) has been shown (Staab *et al*, 1999) by the isolation of a Hwp1 protein encoded by a HWP1 gene expressed in germ-tube and hyphal forms of *C. albicans*. It has been demonstrated that the NH_2-terminus of this protein is a substrate for mammalian transglutaminase, a cross-linking enzyme found in human epithelial and endothelial tissue (Staab *et al*, 1999).

It has also been shown that the *C. albicans* integrin-like Int1 gene/protein is implicated in adhesion. The Int1 protein is a transmembrane protein encoded by the INT1 gene, the deletion of which causes a partial reduction in adhesion to epithelia,

loss of virulence and inhibition of hyphal formation (Gale *et al*, 1998; Calderone & Gow, 2002).

Finally, the ability of *Candida* spp. to form biofilms on the surfaces of medical devices, such as catheters and oral prostheses, has been studied by several investigators (Chaffin *et al*, 1998; Cannon & Chaffin, 1999; Chandra *et al*, 2001; Ramage *et al*, 2001; Calderone & Gow, 2002; Garcia-Sanchez *et al*, 2004). Biofilms seem to confer drug resistance to their inhabitants as the drugs have poor penetration to cells within the biofilms (Kuhn *et al*, 2002a; Kuhn *et al*, 2002b). Microscopy has been used to determine cellular composition during the development of the biofilm (Baillie & Douglas, 1999; Douglas, 2003).

1.2.3 Extracellular hydrolases

Candida spp. produce a range of extracellular enzymes that facilitate adherence and/or tissue penetration. The three most significant hydrolytic enzymes produced by *Candida* species (and most notably by *C. albicans*) are secreted aspartic proteinases (Saps, which hydrolyse peptide bonds), phospholipases (which hydrolyse phospholipids) and lipases (which hydrolyse ester bonds of tryglycerides) (Hube & Naglik, 2002; Naglik *et al*, 2004).

1.2.3.1 Secreted aspartyl proteinases (Sap)

To date, ten members of the SAP gene family, encoding different secreted aspartyl proteinases (Sap 1-10) have been isolated. Many *Candida* species are known to possess SAP genes and produce active extracellular proteinases *in vitro*; and these include *C. albicans*, *C. dubliniensis* (Gilfillan *et al*, 1998), *C. tropicalis* (Togni *et al*, 1991; Monod *et al*, 1994; Zaugg *et al*, 2001) and *C. parapsilosis* (de Viragh *et al*, 1993; Monod *et al*, 1994). The pathogenicity of different *Candida* species may reflect

the ability to produce Saps, and it has been reported that less pathogenic or non-pathogenic *Candida* species produce no Saps or do so in limited amounts (Ruchel, 1992). Furthermore, it has been reported that the success of the fungal pathogen in different types of infection depends partly upon the expression of distinct virulence factors, such as members of the SAP gene family (Hube *et al*, 1997; Sanglard *et al*, 1997; De Bernardis *et al*, 1999b). Whereas SAPs 4-6 have been shown to facilitate systemic infections, the expression of SAPs 1-3 appears important for the virulence of *Candida* in superficial candidosis (Schaller *et al*, 2001). All the secreted *Candida* proteinases belong to the same class of aspartic proteinase (Hube & Naglik, 2002). Extracellular matrices and host surface proteins such as laminin, fibronectin, and mucin are efficiently degraded by Saps, in particular by Sap2, thus aiding yeast adhesion to buccal epithelial cells (BECs). Sap 2 has also been shown to hydrolyse secretory IgA (normally resistant to bacterial proteinases) (Ghannoum & Abu Elteen, 1986; Douglas, 1988) and the action of Sap 2 is greatest at low pH levels (pH 2-5). It has however also been reported that Sap 2 remains stable, and has some activity, at neutral pH levels (Capobianco *et al*, 1992; Wagner *et al*, 1995). It is further known that Sap4 and Sap6 are particularly active at physiological pH levels (pH 5-7) (Hube & Naglik, 2002; Naglik *et al*, 2003). This stability of Saps at different pH levels may assist *Candida* species, in particular *C. albicans*, to colonize and/or infect hosts in a neutral pH environment, such as the oral cavity (Naglik *et al*, 2003; Naglik *et al*, 2004).

1.2.3.2 Phospholipases

Phospholipases are thought to contribute to the pathogenicity of *C. albicans* via hydrolysis of lipids in host cell membranes, particularly during invasion (Mayser *et al*, 1996; Fu *et al*, 1997) and dissemination in the host. It has been proposed that 30-

70% of *C. albicans* isolates exhibit phospholipase activity to varying degrees (Kothavade & Panthaki, 1998). A number of different classes of phospholipase (PL) are known and are classified based on their mode of action (Hube & Naglik, 2002). Phospholipases A, B, C and D (PLA, PLB, PLC, PLD) have been identified in *C. albicans*, but it has been shown that only PLB1 is required for virulence in an animal model of candidosis (Ghannoum & Abu Elteen, 1986; Calderone & Fonzi, 2001). Phospholipases and lysophospholipases may damage host-cell membranes and facilitate *Candida* invasion. Interestingly enough, it has also recently been discovered that non-*C. albicans* species secrete phospholipases, although in smaller quantities compared to *C. albicans* (Ghannoum, 2000).

A correlation between phospholipase activity *in vitro* and virulence has been demonstrated. A study comparing phospholipase activity of *C. albicans* commensal isolates with those from blood revealed that extracellular phospholipase activity was higher in the latter group of isolates, inferring that phospholipases played a role in haematological (and presumably systemic) infection (Ibrahim *et al*, 1995). In addition, the blood isolates had significantly higher rates of germination and their germ tubes were longer than those produced by commensal strains. This study also compared isolates that exhibited high and low phospholipase activity in a murine model of disseminated candidosis. Phospholipase-deficient strains demonstrated a reduced ability to invade and isolates exhibiting increased phospholipase activity caused greater mortality (Ibrahim *et al*, 1995). It would therefore appear that candidal phospholipases may enhance the dissemination of *C. albicans* contributing to the pathogenesis of *Candida* infections (Hube & Naglik, 2002).

1.2.3.3 Lipases

Lipases secreted by *Candida* spp. are able to catalyse the hydrolysis of esters bonds of triglycerides (Hube & Naglik, 2002). Up to now, a family containing 10 lipase genes (LIP1-10) has been identified (Hube *et al*, 2000; Stehr *et al*, 2004) in *C. albicans*. Furthermore, sequences similar to LIP1-10 were also detected in other pathogenic *Candida* spp., such as *C. tropicalis*, *C. parapsilosis* and *C. krusei*, but not in *C. glabrata* (Fu *et al*, 1997; Hube *et al*, 2000; Hube & Naglik, 2002). Different experiments on LIP genes have shown that the transcription of at least some of these genes is lipid-independent and suggest that lipases may have functions other than solely providing nutrients for the cells, *i.e.* contributing to the persistence and virulence of *C. albicans* in human tissue (Hube & Naglik, 2002). It has recently been demonstrated by reverse transcriptase polymerase chain reaction (RT-PCR), that some LIPs of *C. albicans* are expressed constitutively, whilst others are induced under different environmental conditions (Hube *et al*, 2000; Stehr *et al*, 2004). Furthermore, the expression profile of the lipase gene family appears to be dependent on the stage of infection (Stehr *et al*, 2004).

1.2.4 Phenotypic switching

Phenotypic switching, first observed in *C. albicans* as differences in colony morphology (Pomes *et al*, 1985; Slutsky *et al*, 1985; Slutsky *et al*, 1987; Soll, 1992), has also been noted with other *Candida* species, such as *C. glabrata* (Lachke *et al*, 2000), *C. tropicalis* (Soll *et al*, 1988) and *C. parapsilosis* (Enger *et al*, 2001; Soll, 2002). In the 1980s, research demonstrated that low doses of UV light induced a frequent, but reversible transition in *Candida* colony morphology to produce rough colonies (Pomes *et al*, 1985). Correspondingly, another study revealed other colony types, described as "fuzzy", "hat", "stippled" and "wrinkled" (Slutsky *et al*, 1985).

However, the most extensively studied phenotypic switching scheme is the white-opaque (WO-1) system in *C. albicans*, where transition between a hemispherical smooth white colony morphology (white phase) and a flat smooth grey colony morphology (opaque phase) occurs (Slutsky *et al*, 1987; Soll *et al*, 1994; Soll, 2002). Switching regulates a number of phase-specific genes and a high proportion of these genes directly or indirectly influence a variety of virulence traits, such as adhesion (Kennedy *et al*, 1988), yeast-hyphal transition (Anderson *et al*, 1989), secretion of proteinases (Morrow *et al*, 1992; White *et al*, 1993; Monod *et al*, 1994), drug susceptibility (Soll *et al*, 1989) and antigenicity (Anderson *et al*, 1990). Switching occurs both at sites of commensalism and infection, although more frequently in the latter, providing a variety of minor phenotypes for rapid adaptation to immediate environmental challenges (Soll, 2002).

1.2.5 Morphogenesis

Candida albicans is a dimorphic organism that exists in the form of unicellular yeasts (budding cells or blastospores) and filamentous hyphae. Phase transition from yeast to hyphae is termed morphogenesis and is regarded as important in the pathogenesis of infections by *C. albicans* (and *C. dubliniensis*). A recent study established that the ability of *C. albicans* to invade a model of oesophageal candidosis depended upon the ability of the strain to convert from the yeast to the hyphal form (Bernhardt *et al*, 2001). A hyphal-forming strain was able to invade in two days, whereas a strain unable to form hyphae showed no evidence of invasion. The dependence upon filamentation for invasion may be attributed to differences in surface characteristics and antigenicity (Bouchara *et al*, 1990), and morphogenesis may be an important immune evasion mechanism. Yeast cells, as previously reported, are able to invade the pancreas of mice, indicating that yeast cells also possess some invasive potential

(Young, 1958). Hence, morphogenesis can influence pathogenicity of *Candida,* but yeast forms can even cause disease.

1.2.6 Immune evasion

Candida albicans can avoid aspects of the host immune defences using a variety of mechanisms. As mentioned above, *C. albicans* produces proteases that cleave immunoglobulins and can avoid immune elimination via antigenic variation using phenotypic switching and morphogenesis (Naglik *et al*, 2003). However, *C. albicans* can also mask surface antigens by binding to platelets via fibrinogen-binding ligands (Odds, 1994), and has been shown to possess complement binding receptors, considered important in preventing phagocytosis (Heidenreich & Dierich, 1985).

1.3 Clinical aspects of *Candida* infections

Candida spp. cause a broad range of infections, ranging from non-life-threatening mucocutaneous illnesses to invasive processes involving any organ. In this section, particular attention is focused on predisposing factors implicated in the development of oral candidal infections, and on the principal forms of oral candidosis that may be observed in healthy subjects or in patients affected by systemic disease, such as DM.

1.3.1 Host predisposing factors for oral candidosis

Candida spp. are opportunistic oral commensals that can switch to a pathogenic existence resulting in oral candidosis. This commensal-pathogen transition is associated with a number of virulence attributes of *Candida* spp. (Section 1.2), but host factors are also of critical importance in the development of candidal infections. Only when host defences are inadequate can the organism induce infection (Samaranayake, 1990). A wide range of local and systemic host factors has been

implicated in the pathogenesis of oral candidosis (Table 1.1) (Odds, 1988a; Ellepola & Samaranayake, 2000a) and these are discussed in the following sections.

1.3.1.1 Local factors

The integrity of the oral mucosa is essential in preventing the adhesion and penetration of *Candida* spp. into deeper tissue. Although various factors could lead to a breach of oral tissue, trauma caused by natural and artificial teeth is relatively common (Samaranayake, 1990; Scully *et al*, 1994). Dental prostheses, particularly maxillary dentures, can lead to a continuous maceration of the oral fitting mucosa resulting in microscopic breaches of the epithelium (Samaranayake, 1990). In addition, the ability of *Candida* spp. to adhere to the acrylic resins of the dentures, in combination with reduced salivary flow under the prostheses and/or poor hygiene, creates a potential reservoir of yeasts, in contrast to a prosthesis-free oral cavity (Budtz-Jorgensen, 1990b; Radford *et al*, 1999).

Epithelial changes to the oral mucosa, such as atrophy, hyperplasia and dysplasia, may compromise the structure of the mucosal barrier and facilitate candidal invasion, although there are no studies that have compared the thickness of the epithelium in relation to candidal invasion (Samaranayake, 1990; Sitheeque & Samaranayake, 2003).

Adequate salivary flow is essential in preventing oral colonisation and invasion of *Candida* spp. because it removes unattached or poorly adherent *Candida* from the mucosa and inhibits candidal adhesion to host surfaces by the inhibitory action of salivary IgA (Samaranayake, 1990). Furthermore, the presence of lysozyme, a low-molecular weight protein present in high concentrations of saliva and in gingival crevicular fluid, increases *Candida* permeability and stimulates phagocytosis in combination with IgA. Finally, salivary lactoferrin has an antifungal effect through

the sequestering of iron (Soukka *et al*, 1992; Scully *et al*, 1994). It is therefore not surprising that several authors (MacFarlane & Mason, 1974; Martin *et al*, 1981; Samaranayake *et al*, 1984a; Samaranayake *et al*, 1988) have reported that a reduced salivary flow resulting from pathological conditions, such as Sjögren's syndrome or head and neck radiation therapy, may determine an increase in the carriage of oral *Candida* and predisposition to oral candidosis. Furthermore, qualitative changes of saliva, such as high glucose concentration and low pH ranges (pH <5), may also enhance oral candidal carriage, thus promoting subsequent oral infections (Samaranayake, 1990).

There is still controversy regarding the relationship between tobacco smoking and the development of oral candidosis. Some studies (Gergely & Uri, 1966; Bastiaan & Reade, 1982) have suggested that smoking habits do not affect oral *Candida* carriage and predisposition to oral candidosis. However, other authors have reported that smoking may lead to localised epithelial alterations, such as epithelial keratinisation, reduction in salivary IgA and possible depression of the polymorphonuclear leukocyte function, favouring oral candidal colonisation (Zimmermann & Zimmermann, 1965; Kenney *et al*, 1977; Mosadomi *et al*, 1978). In addition, it has been hypothesised that cigarette smoking provides nutrition for *Candida* spp., which are able to enzymically convert some polycyclic aromatic hydrocarbons to produce carcinogens (Hsia *et al*, 1981; Clark *et al*, 1984; Samaranayake, 1990). Furthermore, it has been reported that certain *C. albicans* biotypes are capable of producing carcinogenic nitrosamine N-nitrosobenzylmethylamine from its precursors (Krogh *et al*, 1987). These observations may partly explain the potential role of *Candida* spp. in oral epithelial dysplasia and neoplasia (Scully *et al*, 1994; McCullough *et al*, 2002).

1.3.1.2 Systemic factors

Different systemic conditions have been evaluated as predisposing to oral candidosis. Systemic diseases characterised by quantitative and qualitative immune deficiency (cell-mediated and/or humoral immunity) have been investigated and proved to be involved in the pathogenesis of systemic and mucosal candidosis.

Chronic Mucocutaneous Candidosis (CMC) is a primary immune deficiency in which patients are predisposed to candidal infection which has a characteristic clinical presentation – recurrent and/or recalcitrant candidal infection of mucocutaneous surfaces, as well as a range of haematological, endocrine and neurological complications. It is classified as a distinct primary immunodeficiency, the exact mechanism that predisposes to the candidal infection remain to be determined (Lehner *et al*, 1972; Porter & Scully, 1990; Porter & Scully, 1994b; Lilic & Gravenor, 2001; Lilic *et al*, 2003).

In addition, altered physiological states, such as infancy and old age, in combination with co-factors, such as therapeutic procedures (antibiotic and corticosteroid therapy), congenital defects or local factors, may lead to oral candidal infections (Samaranayake, 1990; Scully *et al*, 1994).

Diabetes mellitus is one of the systemic diseases most frequently implicated with enhanced carriage of oral *Candida* and a higher risk of developing oral candidosis. As this is the principal subject of this thesis, an extensive revision of the relationship between DM and oral candidosis is presented in Section 1.8.9.1.1.

Systemic and/or superficial candidal infection can arise with both solid (*e.g.* lymphoma) and non-solid (*e.g.* leukaemia) malignancies. These candidal infections occur as a result of tumour-related immunosuppresion (*e.g.* neutropenia) as well as therapy that ablates the bone marrow (*e.g.* cytotoxic drug regimes) (Sandford *et al*, 1980; Scully *et al*, 1994; Powderly *et al*, 1995; Feld, 1997; Ruhnke, 2002).

Due to their efficacious anti-inflammatory and immunosuppressive properties, corticosteroids, used systemically or topically, may also result in a lower resistance to infections. Inhaled steroids in particular appear to play a primary role in increasing the risk of oral and oropharyngeal candidosis. Other immunosuppressive regimes (*e.g.* post-allograft receipt) may also predispose to candidal infection – particularly of the mouth (Samaranayake, 1990; Scully *et al*, 1994; Budtz-Jorgensen & Lombardi, 2000).

Broad-spectrum antibiotic therapy is one of the most common iatrogenic factors that is implicated in the development of oral candidosis, altering the local oral microflora and creating a suitable environment for the proliferation of *Candida* spp. It is important to emphasise that local mucosal immunity and normal oral microflora are restored once these therapies are suspended (Budtz-Jorgensen, 1990a; Budtz-Jorgensen, 1990b; Samaranayake, 1990; Ellepola & Samaranayake, 2000a).

Studies show that several nutritional deficiencies are also implicated in the pathogenesis of oral candidal infections. Of these, iron deficiency can cause a series of alterations that may increase the susceptibility of oral mucosa to yeasts. Iron deficiency results in depression of cell-mediated immunity both *in vitro* and *in vivo*, and may determine defects in phagocytosis and inadequate antibody production (Samaranayake, 1986; Samaranayake, 1990; Sitheeque & Samaranayake, 2003).

Other nutritional deficiencies, such as those involving folic acid, vitamins A and B12 are also involved in the causation of oral candidosis often in combination with other systemic and local co-factors (Samaranayake, 1986).

1.3.2 Classification of oral candidosis

Different classifications for oral candidosis have been proposed over the years. The currently accepted classification of oral *Candida* infections is based on two

categories: primary and secondary oral candidosis (Table 1.2). In primary oral candidosis, the oral candidal infection is confined to the oral and perioral tissues whilst oral lesions that are a manifestation of generalized systemic or mucocutaneous candidal infections are classified as secondary oral candidosis (Holmstrup & Axell, 1990; Scully *et al*, 1994). Recently, a proposal for a new classification of oral candidosis has been suggested (Axell *et al*, 1997). The suggestion is that some keratinised primary lesions that are super-infected by *Candida,* such as leukoplakia, lichen planus and lupus erythematosus, may be classified as primary oral candidosis. Examples of clinical presentations of oral candidal infections are shown in Figures 1.3-1.10.

1.3.3 Systemic forms of candidosis

Although less common than oral candidosis, systemic types of candidal infection may manifest themselves in the form of localised primary disease of deep organs or haematogenously disseminated infections (Odds, 1988b). *Candida* spp. are frequently isolated from blood culture in severely immunocompromised patients; the mortality rates from systemic candidosis can be as high as 50% (Romani, 2002). Common risk factors for bloodstream candidosis include extremes of age (low-birth-weight infants and the elderly), immunosuppression, malignancy with leucopenia, central venous catheterisation and prolonged hospitalisation, recently reviewed (Kullberg & Filler, 2002).

Although DM is reported as one of the potential risk factors in the development of candidemia (Kao *et al*, 1999), DM patients are usually not affected by systemic forms of candidosis and for this reason systemic candidal infections are not reviewed in the thesis.

1.4 Treatment of oral candidosis

A variety of antifungal agents with different action mechanisms are available for the treatment of oral *Candida* infections (Table 1.4). The most common antifungal drugs in current clinical use are polyenes (amphotericin B and nystatin), azoles (fluconazole, clotrimazole, miconazole, ketoconazole, itraconazole,) and less frequently, 5-fluorocytosine (5-FC). Recently, a new second-generation triazole (voriconazole) and an echinocandin antifungal agent (capsofungin) have been approved for clinical use by the Food and Drug Association (FDA) (Johnson & Kauffman, 2003). Furthermore, other new triazoles (posaconazole, ravuconazole) are in development for clinical use (Espinel-Ingroff, 1998; Espinel-Ingroff *et al*, 2001; Sanglard & Odds, 2002). In addition, chlorhexidine gluconate has also been proposed (Budtz-Jorgensen & Loe, 1972) as an adjunctive therapeutic supplement for the prevention and treatment of some types of oral candidosis, particularly *Candida*-associated denture stomatitis (Budtz-Jorgensen & Lombardi, 2000).

Ergosterol biosynthesis is specific to fungi and is necessary for their growth. As a result, this feature has been used as the main target in the design of antifungal agents. Among currently used antifungals, only a few (5-FC and echinocandins) do not directly interfere with the ergosterol biosynthetic pathway. The modes of action and resistance mechanisms for each antifungal agent used for the treatment of oral candidal infections are summarised in Table 1.3. Several researchers have, in particular, evaluated the mechanisms responsible for the development of drug resistance in some *Candid*a spp. isolates at the molecular level. For azoles, four distinct resistance mechanisms have been reported (Table 1.3). Up regulation of multidrug efflux transporter genes has been linked with decreased azole susceptibility: these transporters include ATP-binding cassette (ABC) transporter genes (*e.g.* CDR1, CDR2, CDR3, CDR4 from *C. albicans*) (Sanglard *et al*, 1995;

Franz *et al*, 1999; Balkis *et al*, 2002); CgCDR1 and PDH1 from *C. glabrata*; CdCDR1 for *C. dubliniensis*) and major facilitator (MF) genes (MDR1) (Moran *et al*, 1997; Miyazaki *et al*, 1998; Sanglard *et al*, 1999). The most important difference between the two efflux pump systems is that ABC transporters accept almost all the azole agents as substrates, while MF transporters only accept fluconazole as a substrate. In addition, up regulation of ERG11 (a gene encoding for Erg11p, the target of azole derivatives) has been reported as the possible cause of azole resistance in some *C. albicans* and *C. glabrata* isolates. Amino acid substitutions in Egr11p could also affect optimal binding of this target to azole derivatives (Sanglard & Bille, 2002; Sanglard & Odds, 2002).

Recently, the cloning of the gene encoding for a subunit of the β-1,3 glucan synthase in *C. albicans* (CaFKS1) has provided genetic evidence that alterations of this enzyme are implicated in echinocandin resistance, although this is a rare occurrence in comparison with azole resistance (Douglas *et al*, 1997; Frost *et al*, 1997; Mio *et al*, 1997).

1.5 Analysis of *Candida* virulence attributes and associated host responses

1.5.1 Phenotypic methods

1.5.1.1 Adhesion assays

Various methods have been used to examine the adherence of yeast cells to host cells, immobilised protein ligands and plastic surfaces. Typically, the cell-cell adherence assays, using *Candida* spp. and host cells, are either performed in suspension or host cells are grown (or attached) in monolayers to which *Candida* spp. are added (Calderone & Gow, 2002). After rinsing or filtration to remove non-adherent yeast cells, the number of adherent or non-adherent organisms is determined.

Candida adherence to exfoliated BECs as well as vaginal, urogenital and corneal cells (Cannon & Chaffin, 1999; Cotter & Kavanagh, 2000) has been examined in this way. Human embryonic kidney epithelial cells, HeLa (Henrietta Lacks cells - human epithelial cells from a fatal cervical carcinoma infected by Human Papilloma Virus 18- HPV18), fibroblasts, vascular endothelial cells and human umbilical vein endothelial cells have been studied to determine *Candida* adherence (Cotter & Kavanagh, 2000). However, a number of different factors can influence the result of adhesion assays. These factors include the type of fungal strain used and source of the epithelial cells, the number of viable or non-viable BECs and the environment of the yeast culture (*e.g.* temperature and carbon source) (Sandin *et al*, 1987a; Sandin *et al*, 1987b; Polacheck *et al*, 1995). It is also possible to determine the inhibitory action of an agent by first incubating the yeast or host cell/material with the inhibitor in question before the assay.

Candida adherence may be also evaluated microscopically after initial fixation and staining (ruthenium red, crystal violet, Gram stain) of cells (Bernhardt *et al*, 2001; Vitkov *et al*, 2002), or growth (colony forming units - cfu) may act as a measure of adherence by plating cells on agar.

Co-aggregation assays of *Candida* spp., particularly *C. albicans*, with several species of oral bacteria, such as *S. gordoni*, *S. sanguis*, *S. mutans* and *Actinomyces* species have been developed, although the growth conditions for the bacteria can affect co-adherence (Cannon & Chaffin, 1999). Co-aggregation of *C. albicans* and *C. dubliniensis* with oral bacteria, such as *Fusobacterium nucleatum* (one of the most frequent isolate microbes from the subgingival plaque of periodontitis lesions), has recently been described (Jabra-Rizk *et al*, 1999; Calderone & Gow, 2002). These studies are able to identify microorganisms with which *Candida* spp. may interact in the oral cavity. Finally, *in vitro* assays that isolate adhesion by blocking the adherence

of extracted proteins from cells of *Candida*, have also been developed (Edwards *et al*, 1992; Calderone & Gow, 2002).

1.5.1.2 Proteinase assays

Proteinase activity has been primarily investigated using an assay (Macdonald & Odds, 1980) in which *C. albicans* is grown in media to induce proteinase secretion. The culture supernatant is then assayed for its ability to degrade bovine serum albumin (BSA) over a specific period of time (usually 30 min), where cleavage of BSA acts as a measure of enzyme activity. Undigested BSA is removed from the reaction, either by precipitation or filtration, and the amount of digested protein is determined by measuring the optical density at 280nm. Usually, the number of yeast cells per reaction is known, so that enzyme activity is recorded as a change in absorbance per number of yeast cells over a 30 min period. There are different variations of this assay involving the use of proteinase-activity inhibitors such as pepstatin A (Macdonald & Odds, 1980; Kwon-Chung *et al*, 1985; Korting *et al*, 1999) and others (Macdonald & Odds, 1980).

It is well known that Saps are expressed and secreted during different stages and types of *Candida* infections by different *Candida* spp. (Hube & Naglik, 2002; Naglik *et al*, 2003). For this reason, researchers have investigated the *in vitro* SAP gene expression in culture media (Ruchel, 1992; Stewart *et al*, 1999; Monod & Borg-von Zepelin, 2002) and the role of Saps in virulence, using proteinase inhibitors (pepstatin A) and *C. albicans* strains lacking distinct proteinase genes (Fallon *et al*, 1997; Schaller *et al*, 1998).

1.5.1.3 Phospholipase assays

The traditional method of assessing phospholipase activity involves the growth of isolates on Sabouraud's Dextrose Agar supplemented with egg yolk. Phospholipase activity (expressed as a P_z value) appears as a white area of precipitation around each colony, the agar having a turbid appearance (Ibrahim *et al*, 1995; Mayser *et al*, 1996; Fu *et al*, 1997; Kothavade & Panthaki, 1998; Ghannoum, 2000) probably due to the formation of calcium complexes with the fatty acids released by phospholipases acting upon phospholipids in the egg yolk. However, this assay is not specific, as the egg yolk contains substrates from both phospholipases (phospholipids) and lipases (triglycerides), and is not suitable for examining yeast species that produce low levels of phospholipases. Methods for analysing phospholipase activity directly in culture supernatant have also been developed utilizing radiolabelled substrates. Following phospholipase cleavage, radiolabelled products are extracted and initially identified using thin layer chromatography. Liquid scintillation analysis of the separated products then provides an estimate of phospholipase activity. This is expressed as nanomoles of product formed per ml per min or as percentage total of radioactivity per lane (Kothavade & Panthaki, 1998).

1.5.1.4 Organotypic cultures

An organotypic or 'histotypic' culture is the reconstitution of cells to form a three-dimensional tissue structure that mimics the tissue architecture from which the cells originated. For mucosa, the culture comprises fibroblasts dispersed in a collagen gel as a dermal equivalent onto which the epithelial cells are seeded. A variety of epithelial cells may be used, such as keratinocytes or enterocytes, depending on the type of organotypic culture required. Once established, cultures may be infected with microorganisms to produce a model of infection that facilitates examination of

microbial pathogenesis and thereby allows investigation of host-microbial interactions. Methods of analysis involve histopathology and immunohistochemistry, but the cellular response to infection, such as cytokine production, can also be investigated using Enzyme Linked Immunosorbent Assays (ELISAs) or RT-PCR. Organotypic cultures have been used as models of cutaneous candidosis (Korting *et al*, 1998; Dieterich *et al*, 2002) and oral candidosis (Korting *et al*, 1999; Schaller *et al*, 1999a; Schaller *et al*, 1999b) to investigate *C. albicans* adhesion and invasion (Dieterich *et al*, 2002). Organotypic cultures may be an effective alternative to animal models for the investigation of microbial pathogenicity and host microbial interactions. In particular, the model of oral candidosis was considered to morphologically resemble normal oral mucosal epithelium, and, in infection with *C. albicans*, the observed pathology was similar to that observed during natural infection (Schaller *et al*, 1998). However, although organotypic cultures provide a representative model for the investigation of host-microbial interactions, they lack systemic components important in homeostasis, as well as humoral and cell-mediated immune responses, which play pivotal roles in infection control.

1.5.1.5 Animal models

A number of different animal models of candidosis have been employed to investigate host-microbial interactions. Murine and rat models have primarily been utilised for the investigation of systemic, vaginal, cutaneous and oral candidosis (Samaranayake & Samaranayake, 2001). *Candida* production of Saps and SAP expression have been investigated using animal models, particularly regarding their roles in adhesion, invasion and dissemination (Naglik *et al*, 2003). The effects of pepstatin A (Fallon *et al*, 1997) and gene expression during infection (De Bernardis *et*

al, 1995; Staib *et al*, 2000; Naglik *et al*, 2003; Schaller *et al*, 2003) have also been examined.

The virulence and pathogenesis of *Candida* in an animal model may be calculated as a percentage survival rate of animals tested, although conventional histopathological (Fallon *et al*, 1997) or microbiological methods (Calderone & Fonzi, 2001) are also employed. The examination of organs (*e.g.* kidneys, spleen, liver, lungs and heart) can be important in determining tissue tropism and microbial clearance. Although animal models allow the investigation of host microbial interactions, including systemic influences, most of the studies have been performed under different experimental conditions, making them particularly difficult to compare. In addition, *C. albicans* infection of mice has not been reported to occur naturally (Ekenna & Sherertz, 1987). Therefore, extrapolation of findings in animal models to *C. albicans* infection in humans may be limited, as the model does not fully represent the human situation (Schaller *et al*, 1998; Naglik *et al*, 2003).

1.5.1.6 Host immune responses in *Candida* infections

Multiple arms of the immune response act in concert to eliminate infecting organisms. More specifically, phagocytosis, complement and inflammatory mediators have been investigated for their role in *C. albicans* clearance. The ability of polymorphonuclear leukocytes and macrophages to both ingest and kill *C. albicans* blastoconidia has been studied. Previously, a radiometric assay, which measured 3H'uridine incorporation, was used to investigate phagocytosis (Bridges *et al*, 1980). Since a linear relationship exists between uridine incorporation and yeast cell numbers, phagocytosis can be determined by comparing uridine incorporation in a fixed number of *C. albicans* cells with the presence or absence of phagocytes.

Similarly, phagocytic killing can be determined by comparing uridine incorporation after disruption of the phagocyte membrane.

The ability of *C. albicans* to activate complements and its susceptibility to complement-mediated killing have also been investigated (Willcox *et al*, 1998). The susceptibility of *C. albicans* blastoconidia to complement can be evaluated by determining the number of colony forming units (cfu) following incubation of a blastoconidia suspension with human serum. Western blotting can be used to examine complement activation, where the supernatant from a blastoconidia/serum suspension is separated using gel electrophoresis, transferred to a nitrocellulose membrane and probed using anti-human C3 antibodies. This permits identification of C3 fragments, and therefore indicates complement activation.

The ability of *C. albicans* to stimulate production of inflammatory mediators has been examined using ELISAs (Willcox *et al*, 1998). This not only allows cytokine detection, but titration of the culture supernatant allows quantification of cytokine production.

1.5.1.7 Immune evasion

As mentioned previously, *Candida* has a variety of methods for evading host immune responses; these include the binding of platelets to hide surface antigens and phenotypic switching. The ability of *Candida* spp. to bind to platelets has been investigated, as has the effect of inhibitors, using platelet rich and platelet poor plasma (Willcox *et al*, 1998). Platelets and *Candida* cells are mixed in varying ratios, and aggregation is defined as a decrease in optical density. Phenotypic switching can be detected using conventional mycological techniques (Soll *et al*, 1994). Genetic analysis of differential gene expression during transition has been the primary focus

of investigation, particularly on strain WO-1, which switches between a white and opaque phase (Soll *et al*, 1994).

1.5.2 Genetic methods

Phenotypic methods provide an insight into morphological alterations involved in host-microbial interactions, whereas genetic methods elucidate the molecular mechanism behind these interactions.

1.5.2.1 Transcription

The investigation of gene transcription by *Candida* under different conditions has been investigated in an attempt to identify specific genes important for microbial pathogenesis and survival in the host. This can be undertaken by investigating changes that occur in specific known genes, or gene families, or genome-wide variations.

1.5.2.2 Genome-wide transcriptional analysis

Various techniques have been used to assess differential gene expression, including hybridisation screening of cDNA libraries, prepared from extracted RNA of *C. albicans* cells grown under different conditions. Conditions that have been investigated include altered growth temperature, carbon dioxide concentration, type of culture media and pH level. Furthermore, differential hybridisation has been used to study *C. albicans* infection in animal models, yeast budding and hyphal formation (Hoyer *et al*, 1995), as well as phenotypic switching for WO-1 (Morrow *et al*, 1993). A variation of this method using subtractive hybridisation was employed to investigate white opaque switching (Srikantha & Soll, 1993).

More recently, DNA arrays have been constructed and used to examine microbial gene transcription. The DNA microarray is a hybridization-based genotyping technique that offers simultaneous analysis of many polymorphisms. A partial array for *C. albicans* containing approximately 7000 genes was constructed in 2001. This was used to compare gene expression during yeast to hyphal transition of wild types and mutants with changes in pathways important in this transition (Lane *et al*, 2001). Several subsequent studies have used the DNA microarray technique to study different gene expressions associated with *Candida* resistance to antifungal agents (Rogers & Barker, 2002), implicated in *Candida* cell-wall biosynthesis (Sohn *et al*, 2003; Lotz *et al*, 2004) or in adaptation to oxidative stress (Chauhan *et al*, 2003).

1.5.2.3 Gene-specific transcription analysis

The primary method for analysing specific genes or gene families in recent years has been RT-PCR. This involves the extraction of RNA from *C. albicans* grown in different environments, and performing PCR on cDNA synthesised from the RNA using primers specific for the gene or gene family in question. Internal controls may be included, such as actin genes, which allow the evaluation of RT-PCR sensitivity and efficiency (Schaller *et al*, 1998). This method has been applied to examine *C. albicans* SAP gene expression during infection of reconstituted human epithelium and oral biopsy specimens (Schaller *et al*, 1998; Schaller *et al*, 1999a). However, an alternate method for examination of gene expression is the use of promoter-reporter gene constructs. Here, the abundance of reporter product acts as a measure of transcription. This method has again been used predominantly to assess SAP gene expression (Cormack *et al*, 1997; Staib *et al*, 2000).

1.5.2.4 Mutants

An alternative genetic method that assists elucidation of molecular mechanisms of host-microbial interactions is the investigation of specific mutants and their role in pathogenesis. Unlike many other microorganisms, *C. albicans* is diploid. Hence, the construction of mutants requires disruption of both alleles in a gene. The method commonly used for gene disruption in *C. albicans* is the 'URA-blaster' method (Alani *et al*, 1987; Fonzi & Irwin, 1993; Calderone & Fonzi, 2001) that involves the use of a selectable marker (URA3) flanked by direct repeats (bacterial *his*G sequences), whose presence and absence can be selected. SAP mutants have been a particular focus of research using this method. The ability of SAP mutants to adhere and invade reconstructed human epithelium (Schaller *et al*, 1999a), as well as their role in inhibiting phagocytosis and killing in murine macrophages (Borg-Von Zepelin *et al*, 1998) have been studied. However, other *C. albicans* mutants such as the proteinase deficient mutant M12 and transcription factor mutants EFG1 and CPH1 have been investigated (Macdonald & Odds, 1983; Dieterich *et al*, 2002).

Overall *C. albicans* is an exceedingly versatile eukaryotic organism that inhabits a wide variety of niches within the host. Each of these niches exerts different selective pressures upon the organism. Due to this variation and the local or systemic changes in the host's ability to resist infection, the *Candida*-host relationship may be of either a commensal or parasitic nature. The application of both phenotypic and genetic methods to investigate this relationship enhances our understanding of host-microbial interactions. Elucidation of such interactions will ultimately facilitate identification of putative targets for intervention.

1.6 Isolation and identification of *Candida* species from the oral cavity (phenotypic-genotypic methods)

A number of different techniques are proposed for the isolation of *Candida* spp. from the oral cavity (Table 1.6). The choice of method often depends on the type of lesion being investigated (Williams & Lewis, 2000). The oral rinse technique (Section 2.2) involves collecting a 30 s mouthrinse of 10 ml of sterile distilled water (or phosphate-buffered saline). Prostheses should be removed prior to sampling. The solution is then vibrated for 30s for optimal disaggregation; a known volume (100µl) is inoculated onto an agar medium and then incubated for 24-48 h at 37°C. *Candida* growth is expressed in cfu per ml of saliva. This method is rapid, easy to perform and provides quantification of *Candida* and other microorganisms through samples grown in specific culture medium (Samaranayake *et al*, 1986).

1.6.1 Primary culture media

The most frequently used primary isolation medium for *Candida* species is SDA (Odds, 1991), which although permitting growth of *Candida* species, suppresses the growth of many commensal oral bacteria due to its low pH (< pH 6). Bacterial growth can be further suppressed by adding antibiotics (chloramphenicol, penicillin, streptomycin, ciprofloxacin) to the culture medium (Silverman *et al*, 1990). Plates can either be incubated aerobically at 37°C for 24-48 h or at room temperature for 2-3 days; *Candida* develop in the form of cream-coloured, convex colonies and may have a fringe of submerged hyphae. It should be noted, however, that coliforms which sometimes grow on SDA, may mimic yeasts in appearance (Silverman *et al*, 1990). SDA rarely permits differentiation between *Candida* species within the same sample. It is in fact estimated that more than one *Candida* species occurs in approximately 10% of oral samples and consequently, it is recommended that SDA should be used in combination with other culture media (MacFarlane, 1990).

Examples of other culture media used for the isolation and identification of *Candida* spp. are reported in Section 1.6.2.1.

1.6.2 Identification of *Candida* species from the mouth - Phenotypic Methods

As described previously, there is increasing diversity of *Candida* isolates from the mouth and so consequently, there is a need not only detect to *Candida*, but also to identify the isolated yeast. Different systems of identification based on phenotypic properties of *Candida* have been proposed. Although they are simple and have been applied in epidemiological studies, it is not always possible to accurately repeat the techniques.

1.6.2.1 Direct identification on primary culture media

The incorporation of fluorogenic or chromogenic substrate directly into the growth agar media to reveal species-specific enzyme activities allows discrimination of particular *Candida* species colonies in a mixed yeast population, and thus allows rapid identification of *Candida* species on the primary isolation medium (Freydiere *et al*, 2001). Chromogenic substrates are now preferred to fluorogenic substrates due to their stability and ease of interpretation. Chromogenic substrates are also commercially available. Of note, CHROMagar® *Candida* (CHROMagar, Paris, France) can detect and differentiate between *C. albicans, C. tropicalis, C. krusei* and *C. dubliniensis,* based on colony colour and appearance, while other chromogenic media identify only *C. albicans* (Freydiere *et al*, 2001).

CHROMagar *Candida* can differentiate *C. albicans* from *C. dubliniensis* (Sullivan *et al*, 1995), as the latter appear as darker green colonies. However, this differentiation appears to decline after isolate and subsequent subculture. Failure of *C. dubliniensis* to grow on agar media at the elevated incubation temperature of 45°C has recently

been suggested as an alternative test (Odds & Bernaerts, 1994; Schoofs *et al*, 1997; Pinjon *et al*, 1998; Williams & Lewis, 2000). Recently, it has been shown that the speed of assimilation of some sugars achieved with rapid commercial systems allows differentiation between *C. albicans* and *C. dubliniensis* (Freydiere *et al*, 2001). An anti-*C. albicans* cell wall surface-specific monoclonal antibody has been described as useful for differentiating between the two species (Marot-Leblond *et al*, 2000).

In contrast to the findings of others (Pfaller, 1996; Willinger & Manafi, 1999), some authors (San Millan *et al*, 1996; Powell *et al*, 1998; Hoppe & Frey, 1999; Freydiere *et al*, 2001) report that CHROMagar *Candida* does not allow the identification of *C. glabrata* and other emerging pathogens that have different susceptibility levels to antifungal agents. To avoid misidentification, it is thus important to establish the limitations of the methods and reagents used for fungal identification.

Pagano Levin agar (Pagano *et al*, 1957), which is not commercially produced, distinguishes yeast species on the basis of differences in colour, which are attributed to a reduction in triphenyltetrazolium chloride incorporated in SDA. This medium produces pale coloured colonies of *C. albicans*, while colonies of other *Candida* species produce colonies in varying degrees of pink (Samaranayake *et al*, 1987).

1.6.2.2 Germ-tube test

This is the standard laboratory method for differentiating *C. albicans* from other *Candida* species; however, *C. dubliniensis* is now also known to produce germ-tubes (Sullivan & Coleman, 1997; Sullivan & Coleman, 1998). The test involves the rapid induction of hyphal outgrowths (germ-tubes) from yeasts cultured in serum (rabbit, bovine, horse or pooled human sera) for 2-4 h at 37°C. Following incubation, suspensions are placed on a glass slide and examined microscopically for the presence of germ-tubes. A germ-tube is a filamentous, cylindrical hyphal outgrowth

from the yeast cell with no constriction present at the base (Odds, 1988c; Silverman *et al*, 1990; Williams & Lewis, 2000) (Fig.1.1). The formation of germ-tubes is influenced by different factors, *e.g.* temperature, inoculum size, composition of the medium and *C. albicans* strain (Mackenzie *et al*, 1962).

1.6.2.3 Chlamydospore formation

Similar to germ-tube production, chlamydospore formation is a peculiar ability observed in *C. albicans*, *C. dubliniensis* (Sullivan & Coleman, 1998) and occasionally in isolates of *C. tropicalis* (Hasenclever, 1971; Silverman *et al*, 1990; Williams & Lewis, 2000). The chlamydospore has been defined as a large, refractile, thick-walled cell induced *in vitro* by culture on agar supplemented with cornmeal Tween 80 (Fig. 1.2). Following incubation for 1-2 days at room temperature, chlamydospore growth can be observed by direct microscopy (Odds, 1988c; Silverman *et al*, 1990; Williams & Lewis, 2000).

1.6.2.4 Physiological tests

The physiological properties of *Candida* species often need to be related to the morphological criteria. The main tests used in the identification of different species involve the examination of their ability to assimilate and ferment individual carbon and nitrogen sources (Silverman *et al*, 1990) by inspecting zones of *Candida* growth around disks or wells impregnated with various sugars in basal agars (Di Menna, 1955). It is also possible to evaluate *Candida* growth in tubes containing synthetic basal medium (yeast nitrogen base) to which different carbon sources are added.

1.6.2.5 Rapid commercial identification systems

The introduction of rapid commercial identification systems increases the accuracy of *Candida* species' identification. These systems are based on growth (relatively slow, 72 h of incubation) and enzyme production profiles (results within a few h). The analysis can be performed manually or using automated systems (Table 1.7).

1.6.2.6 Histopathological appearance of *Candida* species in tissue

Candida species can be identified histopathologically in appropriate stained (e.g. Periodic Acid Shiff, PAS) tissue specimens from superficial or systemic sites, as yeasts and pseudohyphae, or yeast cell alone. *Candida albicans* may be distinguished from other yeasts by the presence of yeast cells plus hyphae and/or pseudohyphae (Richardson & Carlson, 2002).

1.6.2.7 Yeast killer toxin typing

A *Candida* strain differentiation system based on the susceptibility of *Candida* isolates to yeast killer toxins, has been reported (Polonelli *et al*, 1983; Caprilli *et al*, 1985). *Candida* isolates are grown with killer toxin-producing yeast strains (direct technique) (Section 1.7) in modified agar containing glucose and peptone. Following incubation, toxins released in the agar medium inhibit the growth of *Candida* test strains (Polonelli *et al*, 1983; Silverman *et al*, 1990). A method using purified yeast killer toxins (particularly ones produced by the genera *Pichia*) in place of killer yeast cells can also be used for differentiating strains of *Candida* isolates (indirect technique). A computer-aided system can be used to evaluate, record and compare the halo of inhibition displayed by each killer toxin on each susceptible strain (Polonelli & Morace, 1986a; Polonelli & Morace, 1986b; Magliani *et al*, 1997b).

1.6.3 Identification of *Candida* species - Genotypic Methods

Recent reports indicate an increasing number of pathogenic non-*C. albicans* species such as *C. dubliniensis*, *C. glabrata* and *C. krusei*, possibly selected for as a result of widespread introduction of antifungal drugs (Nguyen *et al*, 1996; Pfaller *et al*, 1998a; Pfaller *et al*, 1999a; Sullivan & Coleman, 2002). Routine standard methods of identification, including commercial automated systems, are not always able to analyse and identify these species, and subsequent treatment may be inappropriate. Correct identification is important to establish whether recurrent infections are due to a novel organism or the persistence/re-infection of the original strain and whether specific clones or clusters of related strains develop a resistance to drugs (Sullivan & Coleman, 2002). The accurate differentiation of species and strains requires the implementation of molecular biological methods that are mainly based on different PCR techniques. It is generally accepted that genetic methods of strain delineation are sensitive, reproducible and have relatively high discriminatory powers (Wilson *et al*, 2001) and thus are proposed to be more advantageous than phenotypic methods. However, genetic methods can be time-consuming, laborious, costly and may require specialized equipment (Lehmann *et al*, 1992). Table 1.7 summarises the most important molecular biology techniques described in literature for *Candida* identification. Details of these techniques can be found elsewhere (Soll, 2000; Dassanayake & Samaranayake, 2003b; Gil-Lamaignere *et al*, 2003). The following discussion details the molecular biological techniques directly employed or strictly related to those adopted in this study for the identification of *Candida* spp. or analysis of genetic differences within a *Candida* spp.

1.6.3.1 Polymerase Chain Reaction-based typing

Different PCR techniques have been used to identify *Candida* species, based on specific candidal genes. Ribosomal DNA (rDNA-genes encoding for ribosomal RNA) is a frequent target in PCR systems as multiple copies of rDNA sequences exist within the genome and it is more sensitive. One of the most frequently used methods is based on the analysis of repeated sequences (PCR amplicons) of rDNA of *Candida* species achieved by restriction fragment length polymorphisms (RFLPs).

To differentiate between all medically important species of *Candida*, PCR primers have been directed against the V3 region of the 25S rDNA and against specific sites of the Intergenic Transcribed Spacer region (ITS) of *Candida* species. Amplicons of V3-25S and ITS PCR are then digested with specific restriction endonucleases, *e.g.* *Hae* III and *Dde* I, respectively. The resulting restriction fragments are then analysed by agarose gel electrophoresis. This technique has been used by several authors (Williams *et al*, 1995; McCullough *et al*, 1999a) to identify a number of medically relevant important *Candida* species, such as *C. glabrata, C. krusei, C. parapsilosis, C. guilliermondii, C. tropicalis, C. lusitaniae, C. pseudotropicalis* and *C. dubliniensis*, based on size and primary structural differences in the rDNA regions. This method has shown that one of the *Candida* species, *C. stellatoidea*, is synonymous with *C. albicans* (genotype B) (McCullough *et al*, 1999b).

There are a number of PCR-based methods that have been used for *C. albicans* strain differentiation, including random amplified polymorphic DNA (RAPD), inter repeat PCR (IR-PCR) and microsatellite analysis, often collectively referred to as PCR fingerprinting. Random amplified polymorphic DNA (RAPD), or arbitrary primer PCR (AP-PCR) analyse short regions of DNA sequence for variation based on PCR priming regions. These PCR methods are based on the use of one short PCR oligonucleotide primer (about 10-15 bp) of arbitrary sequence and with low

stringency amplification reaction conditions. Due to the low annealing temperatures used, primers can bind to many sites throughout the genome, thus allowing the amplification of products that vary in size. When separated by agarose gel electrophoresis and subsequently stained with ethidium bromide, the fragments obtained from PCR can yield strain-specific fingerprint patterns comprising 3-6 bands (Sullivan & Coleman, 2002). If a comparison of several isolate amplifications shows a band (locus) that varies, alleles are assigned to the presence (1) or absence (0) of the band (Taylor *et al*, 1999).

Although these techniques are methodologically simpler, less time-consuming and more cost-effective than other genomic typing methods, they are limited in their ability to identify significant problems regarding the reproducibility of typing results (Taylor *et al*, 1999).

1.6.3.2 PCR fingerprinting with repetitive DNA sequences

In this PCR technique, the primers recognise a repeat DNA sequence, producing a large number of fragments with each reaction. The primers are longer (16 to 28 bp) than those used in RAPD and the annealing temperature used is higher to avoid reproducibility problems. In comparison with other strain-typing methods, PCR-fingerprinting techniques are usually the most differentiating, simple and reproducible (Diaz-Guerra *et al*, 1997; Taylor *et al*, 1999) but due to the possible homology of bands, estimating a genetic relationship between groups of strains is problematic and it is difficult with PCR-fingerprinting techniques to establish the precise degree to which strains differ.

1.7 Yeast killer phenomenon

Yeast killer toxins (KTs) are exotoxins (generally proteins or glycoproteins) that are secreted naturally by many different yeasts and are capable of killing susceptible cells belonging to the same or congeneric species (Somers & Bevan, 1969; Young & Yagiu, 1978; Wickner, 1985; Polonelli *et al*, 1991b; Magliani *et al*, 1997b). This phenomenon, first reported in *Saccharomyces cerevisiae* (Bevan & Makower, 1963) has been described in different yeasts and genera, such as *Kluyveromyces, Ustilago*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Pichia*, *Torulopsis*, and *Williopsis* amongst others (Philliskirk & Young, 1975; Wickner, 1985; Young, 1989). The killer phenotype is genetically encoded by cytoplasmatic viral double stranded RNA (dsRNA) or linear DNA plasmids as well as chromosomal genes and is mediated by specific cell-wall receptors in sensitive yeasts (Stumm *et al*, 1977; Bussey, 1981; Hutchins & Bussey, 1983; Tipper & Bostian, 1984; Schmitt & Radler, 1987; Starmer *et al*, 1987; Sawant & Ahearn, 1990; Kimura *et al*, 1993; Kasahara *et al*, 1994). The *Pichia* and *Williopsis* killer systems, produce different killer toxins, some of which interfere with the synthesis of β-1,3-D-glucan, the major cell-wall polysaccharide polymer involved in determining cell morphology and maintaining osmotic integrity (Magliani *et al*, 1997b). In particular, HM-1 (or HMK), one of the most studied killer toxins produced by *Williopsis mrakii*, a basic unglycosylated polypeptide of 10.721kDa, shows high thermostability (100°C for 10 min) and a pH stability between pH 2 and 11 (Yamamoto *et al*, 1986). It has been reported that HM-1 is able to kill susceptible strains of *S. cerevisiae* and other yeasts, by firstly interfering with the synthesis of β-1,3-D-glucan, thus rendering the cell wall osmotically fragile and subsequently resulting in lytic cell death (Magliani *et al*, 1997b). This mechanism is similar to that of antifungal antibiotics such as aculeacin A, echinocandin B and papulocandin B (Yamamoto *et al*, 1986).

Killer cells are immune to their own toxins, and the exact mechanisms of killing and immunity still remain unknown for some killer systems (Magliani *et al*, 1997b).

Interest in the yeast killer phenomenon has increased particularly because of the surprising susceptibility of microorganisms of clinical interest, such as *C. albicans*, *Pneumocystis carinii* and *Mycobacterium tuberculosis*, to killer toxins from species of the genera *Pichia* and *Williopsis* (Polonelli & Morace, 1986a; Morace *et al*, 1989; Aliouat *et al*, 1993; Magliani *et al*, 1997b). The biological activity of a toxin produced by the killer strain *P. anomala* ATCC 96603 (PaKT), a large glycoprotein encoded by nuclear genes, has been studied against susceptible microorganisms, such as *Candida*. Susceptible organisms to this toxin are characterized by the presence of specific cell wall receptors, most likely β-glucans (Polonelli & Morace, 1986b; Polonelli *et al*, 1990). The wide spectrum of activity has led to the consideration of PaKT as a potential therapeutic agent in the treatment of fungal infections (Polonelli *et al*, 1986). Regretfully, PaKT has proven to be toxic and very labile at physiological pH and temperatures necessary for systemic antifungal therapy (Pettoello-Mantovani *et al*, 1995; Conti *et al*, 1996). It has been also reported that yeast killer toxins will not be as effective as antibiotics because of their toxicity and strong antigenicity, features that are not unsurprising considering that these molecules represent large foreign glycoproteins.

In order to exploit the antimicrobial activity of PaKT without inducing undesired effects, KT-neutralizing monoclonal antibody (mAbKT4) was produced according to idiotypic network theory (Jerne, 1974). To obtain the large amounts of standardized reagents required for therapeutic purposes and still exploit PaKT's antimicrobial activity without undesired effects, anti-idiotypic antibodies (KT-IdAbs) have been produced. These molecules represent the internal image of PaKT and are produced in the monoclonal (mAbKT) and recombinant format (KTscFv) using a PaKT-

neutralizing monoclonal antibody (mAbKT4) as an immunogen (idiotypic vaccination) (Polonelli & Morace, 1987; Magliani *et al*, 1997a; Polonelli *et al*, 1997). Subsequent Killer toxin monoclonal antibodies (mAbK10) and KTscFv (scFvH6) have displayed a microbicidal activity *in vitro* and a therapeutic effect *in vivo* against killer toxin receptor (KTR)-bearing eukaryotic and prokaryotic pathogenic microorganisms (Magliani *et al*, 1997a; Seguy *et al*, 1997; Conti *et al*, 1998; Conti *et al*, 2000; Cenci *et al*, 2002; Conti *et al*, 2002; Savoia *et al*, 2002).

1.8 Diabetes Mellitus and its association with oral candidosis

Diabetes mellitus is a complex multisystemic disorder characterized by a relative or absolute deficiency of insulin secretion and/or concomitant resistance to the metabolic action of insulin on target tissues (Garber, 1998). Hyperglycaemia is the immediate metabolic consequence of DM but ultimately, widespread multisystem damage can occur, *e.g.* microvascular disease (microangiopathy) with capillary basement membrane thickening, macrovascular disease (macroangiopathy) with accelerated arteriosclerosis, neuropathy involving both the somatic and autonomic nervous systems, neuromuscular dysfunction, embryopathy, and decreased resistance to infection (Garber, 1998).

Diabetes mellitus can have a variable, and sometimes, profound effect upon oral tissues. This is typified by patients with poor glycaemic control being particularly prone to severe and/or recurrent bacterial or fungal infections.

1.8.1 Classification and pathogenesis of DM

A number of different classification systems have been proposed for DM (National Diabetes Data Group, 1979; World Health Organization, 1985). The American Diabetes Association has recently described a classification system based upon

disease etiology (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998; American Academy of Periodontology, 1999). The classification scheme includes two major forms of DM, termed type 1 (previously insulin-dependent DM or IDDM), and type 2 (previously non-insulin-dependent DM or NIDDM) DM (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998).

1.8.2 General signs and symptoms of DM

The initial clinical features of type 1 DM include the typical triad of polyuria, polydypsia and polyphagia. Irritability, malaise, apathy and pruritus can also be early features of type 1 DM (Teuscher *et al*, 1989; Rees & Otomo-Corgel, 1992; Nathan, 1993; Rees, 1994; Bell & Hockaday, 1996; Garber, 1998; The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998). Patients with undiagnosed type 1 DM are prone to ketoacidosis (Bell & Hockaday, 1996; The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998). The features of type 1 DM are reversible with effective insulin therapy. The features of type 2 DM are of slower onset and less specific than those of type 1 and are sometimes only detected when patients are examined for diseases unrelated to DM. Patients with type 1 and type 2 DM have an equal risk of developing vascular complications.

1.8.3 Complications of DM

While good glycaemic control can prevent or reduce the likelihood of possible complications linked to DM, approximately 50% of patients with DM develop chronic vascular complications following years of DM (Rees, 1994). These chronic

complications of DM are summarised in Table 1.8 (Teuscher *et al*, 1989; The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998).

1.8.4 Type 1 DM

Type 1 DM immune-mediated (A) (previously termed juvenile-type onset diabetes or insulin dependent) constitutes 5% to 15% of all cases of DM. Although this condition is prevalent on a global scale, North Americans and Europeans are more affected than other ethnic groups (Atkinson & Maclaren, 1994; Winter, 1996). This type of DM is caused by cell-mediated autoimmune destruction of the β-cells on the islet of Langerhans in the pancreas (Atkinson & Maclaren, 1994), leading to a complete inability of the cells to secrete insulin. Type 1 DM usually develops before 30 years of age, although can occur at any age. It is suggested that pancreatic destruction occurs when genetically predisposed individuals are subjected to a triggering event, such as a viral infection that induces a destructive autoimmune response (Smith, 1987; Rees, 1994). The rate of β-cell destruction is variable, being rapid in some individuals (usually infants and children) and slow in others (typically adults) (Zimmet *et al*, 1994). There are two age-associated peaks of incidence, most commonly halfway through the first ten years of age, and during adolescence. It has recently been suggested that β-cell damage is caused by islet cell autoantibodies (ICAs), autoantibodies to insulin (IAAs), autoantibodies to glutamic acid decarboxylase (GADA65) and autoantibodies to the tyrosine phosphatases IA-2 and IA-2β. Eight-five to ninety percent of individuals with initial hyperglycaemia have one or more of these autoantibodies, which can be considered as markers for this type of DM (Cantor *et al*, 1995; Atkinson & Eisenbarth, 2001; Bingley *et al*, 2001).

The idiopathic form of type 1 DM (B) is of unknown aetiology. Most affected patients have permanent insulinopenia and are prone to ketoacidosis. This form of

DM is strongly inherited, but lacks immunological evidence for β-cell autoimmunity and is not HLA associated (Banerji & Lebovitz, 1989; The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998).

1.8.5 Type 2 DM

Type 2 DM, previously termed non-insulin-dependent DM, often occurs in middle to late life and is the more common form of DM, accounting for 80% to 93% of all DM patients (National Diabetes Data Group, 1985; American Academy of Periodontology, 1999). Type 2 DM is characterized by β-cell dysfunction in secreting adequate amounts of insulin, particularly after meals, and/or peripherical insulin resistance. Patients with type 2 DM have some endogenous insulin secretory capability, but have overt abnormalities of glucose homeostasis, including fasting hyperglycaemia (Reaven *et al*, 1976; Turner *et al*, 1979; Bell & Hockaday, 1996; Garber, 1998). Depending on the degree of accompanying hyperglycaemia, patients with type 2 DM are managed by dietary control of sugars and/or with oral hypoglycaemic agents, although sometimes insulin therapy becomes necessary if their disease cannot be managed adequately with oral agents and diet.

Type 2 DM frequently remains undiagnosed for many years and in the early stages of disease, hyperglycaemia develops gradually and is often not severe enough to give rise to polyuria, polydypsia or weight loss (Fujimoto *et al*, 1987; Harris, 1989). Unlike patients with the type 1 disease, those with type 2 DM are relatively resistant to the development of ketoacidosis as a direct consequence of the retention of endogenous insulin secretion. Type 2 DM often has a familial basis, although does not clinically manifest until middle to late life. As a consequence of an accompanying resistance to insulin, obesity is a major risk factor in DM, indeed up to 80% of patients with type 2 DM have mild to marked obesity (Zimmet, 1992; Harris *et al*,

1995). Women with prior gestational DM (GDM) may also be prone to type 2 DM as are individuals with hypertension or hyperlipidemia (Barnett *et al*, 1981; Newman *et al*, 1987).

1.8.6 Diagnostic criteria for DM

The diagnostic criteria for DM as recommended by the National Diabetes Data Group (National Diabetes Data Group, 1979) and the World Health Organisation (World Health Organization, 1985) have been modified recently by an Expert Committee (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998) and are reported in Appendix 1.

1.8.7 Assessment of glycaemic control in patients affected by DM

Different methodologies for assessing glycaemic control are available, depending on the severity of the disease and the clinical setting (Table 1.9). Of these, the estimation of the blood level of glycosylated haemoglobin ($HbA1_c$) provides an accurate and objective measure of glycaemic control over past weeks to months. The rate of formation of this $HbA1_c$ is directly proportional to the ambient blood glucose concentration. Glycosylated haemoglobin is expressed as a percentage of the normal haemoglobin. Non-DM subjects have $HbA1_c$ values of less than 6%, while levels in poorly controlled patients may reach 10 to 12%, and can be as high as 20%. In clinical practice, $HbA1_c$ is usually measured periodically (at least biannually) to assess glycaemic control, to permit appropriate changes to treatment and to determine the degree of inconsistency with a patient's records of home blood glucose monitoring (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998; Goldstein *et al*, 2003).

1.8.8 Management of DM

The aims of DM management are to maintain normal blood glucose levels without episodes of hypoglycaemia, and to prevent or reduce complications caused by long-standing disease. These aims cannot be achieved without good patient cooperation.

1.8.8.1 Oral hypoglycaemic agents and insulin

Drugs commonly used to manage type 2 DM are oral hypoglycaemic agents, particularly sulphonylureas and biguanides. Sulphonylureas stimulate the secretion of insulin and increase the number of insulin receptors if there is some endogenous insulin production. Examples of currently employed sulphonylureas are shown in Table 1.10. Metformin is the only biguanide available. This drug acts mainly by decreasing hepatic gluconeogenesis and increasing peripheral utilisation of glucose. A number of other available agents for managing DM appear in Table 1.11. Newer anti-diabetic drugs are usually prescribed in combination with sulphonylureas and biguanides when older agents prove inadequate in reducing high blood-glucose levels.

Insulin therapy is required for type 1 DM, and for patients with long-standing type 2 DM when other therapies prove unsuccessful. Insulin is administered by subcutaneous injection and is available in short-acting (for 2 to 6 h), intermediate-acting (1 to 20 h) and long-acting (over 40 h) forms (Mealey, 1998). Mixed insulin preparations are also available. Management typically involves a combination of short-acting and intermediate-acting insulin (Table 1.11). Continuous subcutaneous insulin infusions or "insulin pumps" are now available. These deliver a basal dosage of insulin to maintain glucose control without hypoglycaemia (Varon & Mack-Shipman, 2000).

Insulin is available in three different forms: human (produced synthetically or by DNA recombinant technology using *Escherichia coli*), porcine and bovine. Human insulin has a more rapid onset and shorter duration than porcine insulin, while bovine insulin has the longest duration of activity (Mealey, 1998), although the non-human forms are now rarely employed.

The absorption of insulin from subcutaneous sites is variable both within and between individuals and therapy needs to be tailored to suit the life-style of the patient (Bell & Hockaday, 1996). The main aim of insulin therapy is to reproduce the natural peak levels of insulin during and after meals post-prandially using a basal concentration. The pharmacokinetics of insulin make this difficult and hypoglycaemia is the most common complication of inadequate management (Bell & Hockaday, 1996). Detailed descriptions of insulin therapy can be found elsewhere (Garber, 1998).

1.8.9 Oral disease manifestations associated with DM

A wide spectrum of oral manifestations of DM is discussed in the following sections.

1.8.9.1 Fungal infections

1.8.9.1.1 Oral candidosis and other candidosis

Several authors have reported that DM, especially when poorly controlled, may predispose to a variety of superficial and systemic fungal infections (Fisher *et al*, 1987; Hill *et al*, 1989; Lamey *et al*, 1992; Finney *et al*, 1997; Guggenheimer *et al*, 2000b; Lalla & D'Ambrosio, 2001), particularly oral candidal infections, whose clinical course can be more severe (*i.e.* recurrent and chronic) than that of patients without DM. However, although the majority of reports show a correlation between *Candida* and DM (Ueta *et al*, 1993; Guggenheimer *et al*, 2000b), the notion that candidal infection is more severe or frequent in DM sufferers than in healthy individuals remains unresolved (Samaranayake, 1990; Vazquez & Sobel, 1995).

It also remains unclear as to whether the candidal load is a true reflection of the likelihood of a clinical manifestation of fungal disease and if an increased *Candida* oral load promotes systemic infection. A number of specific studies have attempted to establish cut-off limits for cfu in order to distinguish between carriers and patients with oral candidosis (Samaranayake *et al*, 1986) but it is evident from literature (Bartie *et al*, 2004) that checks can reveal higher levels of *Candida* (> 9 X 10^3 cfu/ml) in healthy individuals than in patients affected by oral candidal infection.

Candida albicans is the most prevalent *Candida* species isolated from the oral cavity of both DM and healthy individuals (Dorocka-Bobkowska *et al*, 1996; Willis *et al*, 1999). *Candida dubliniensis*, a recently discovered species of *Candida* that was mostly isolated from the oral cavities of HIV-infected patients, was detected in 58 out of 318 insulin-treated DM patients (Willis *et al*, 1999). *Candida dubliniensis* has also been isolated from the oral cavity of patients with both type 1 and type 2 DM (Manfredi *et al*, 2002) and seems to have a predilection for dentate patients. The pathogenic significance of this new species in the oral cavity of patients with DM remains unknown. It has been established that *C. dubliniensis* usually co-colonizes the oral cavity with *C. albicans* or with other *Candida* spp. (Willis *et al*, 2000b). Whether this co-colonisation has any influence on clinical outcomes is yet to be determined.

The frequency and density of *Candida* colonisation and the development of oral candidosis in DM patients appear to result more from a combination of host and fungal risk factors (*e.g.* degree of glycaemic control, presence of dentures, smoking habits, age, medication, adherence of *Candida* spp.), rather than any other single factor (Darwazeh *et al*, 1990; Darwazeh *et al*, 1991b).

As with healthy individuals, there is great variation in the isolation rates of oral *Candida* in patients affected by DM (Table 1.12). These differences are often due to

the diversity of sampling techniques used by the authors to quantify oral yeast carriage. However, a number of studies have shown that the oral rinse sampling method (Samaranayake *et al*, 1986) is the most appropriate and sensitive technique for evaluating overall oral *Candida* carriage. A recent study (Manfredi *et al*, 2002), using this methodology found that patients with DM are no more likely to harbour yeast in their oral cavity than healthy individuals, thus DM itself may not place a person at increased risk of fungal carriage or clinical infection, although poor diabetic control and a high level of glycosylated haemoglobin (>12%) (Hill *et al*, 1989; Vazquez & Sobel, 1995) may increase susceptibility to fungal infection (Gibson *et al*, 1990; Ueta *et al*, 1993; Willis *et al*, 2000b).

Furthermore, it must be noted that different DM populations (in terms of type of DM or diabetic treatment) have been examined in different studies over the years and different variables evaluated (*e.g.* glycaemic control), thus rendering the comparison between these studies quite difficult.

There is no clear mechanism by which DM may predispose to higher oral candidal carriage rates than in healthy subjects. However, it is well known that high salivary glucose levels in patients affected by DM favour yeast growth (Knight & Fletcher, 1971). The hyperglycaemia of poorly controlled DM may influence the pathogenic colonisation of *Candida* (Samaranayake & MacFarlane, 1982). Yeast growth and adhesion may be enhanced by high glucose concentration (Samaranayake *et al*, 1984b) in blood (Odds *et al*, 1978) and in saliva (Knight & Fletcher, 1971), which can serve as a nutrient for *Candida*.

The reduced capacity for killing *Candida* by neutrophilis (Hostetter, 1990; Ueta *et al*, 1993; Vazquez & Sobel, 1995) in the presence of high glucose concentrations may also account for any increased colonisation by *Candida*. Reports show that polymorphonuclear leukocyte activity in DM patients (with high salivary and blood

glucose levels was reduced) with oral candidosis (Ueta *et al*, 1993). There was also a concurrent reduction in phagocytosis and intracellular killing of *Candida* cells compared with non-DM subjects or DM patients with good metabolic control.

In vitro studies have demonstrated that in type 1 DM, the adhesion of *Candida* seems to be higher to palatal cells than buccal mucosal epithelial cells. The adhesion of *Candida* strains to oral mucosa seems to be influenced by the type of sugars in the culture medium and by the *Candida* strain, which could modify its surface composition in response to high salivary glucose concentration (Willis *et al*, 2000a). It has also been reported that the adhesion of *Candida* to BECs collected from patients affected by DM is significantly greater than adhesion obtained from BECs from non-DM controls (Darwazeh *et al*, 1990). These results have also been reported by others (Dorocka-Bobkowska *et al*, 1996) who stated that type 1 DM patients had a greater mean candidal load than non-DM subjects. Thus in DM patients, intrinsic qualitative changes on cell surface receptors may modulate yeast adhesion (Samaranayake, 1990). Salivary glucose, in particular, may form chemically reversible glycosylation products with proteins in tissues during hyperglycaemic episodes (Brownlee *et al*, 1988). It has been postulated that the accumulation of these glycosylation products on BECs may increase the number of available receptors for *Candida* cells.

In addition, it has been reported that DM patients are prone to developing xerostomia (Section 1.8.9.3) for a number of different reasons, such as decreased salivary function and reduced salivary flow rate caused by microvascular disease and neuropathy dysfunction affecting the minor/major salivary glands. It is well known that a reduction in salivary flow and associated salivary components (sIgA, lysozyme, lactoferrin) inhibitory to *Candida* adhesion to BECs, could promote candidal colonisation (Clowes *et al*, 1978).

It has been suggested that oral carriage loads of *Candida* species may be influenced by the DM type. However, there is no confirmed association between oral candidal carriage and DM type or severity. The different reported rates of oral carriage of *Candida* obtained (Barlow & Chattaway, 1969; Aly *et al*, 1992; Bai *et al*, 1995; Manfredi *et al*, 2002) may reflect the different sample sizes or differences in the sample populations (e.g. age, diabetic therapies, denture status) rather than any influence from DM.

Indeed, denture wearing may influence yeast colonisation in patients with DM more than any systemic aspect of DM. Several studies have reported a high prevalence of *Candida* carriage in DM patients using removable dentures (Tapper-Jones *et al*, 1981; Fisher *et al*, 1987; Lamey *et al*, 1988; Hill *et al*, 1989; Aly *et al*, 1992; Dorocka-Bobkowska *et al*, 1996; Willis *et al*, 1999). It is well recognised that *Candida* spp. are able to adhere not only to host mucosa but also to acrylic surfaces, which could act as a reservoir for microorganisms, especially when the dentures are ill-fitting and not routinely cleansed (Samaranayake & MacFarlane, 1980; Samaranayake *et al*, 1980; Ellepola *et al*, 1990; Ellepola & Samaranayake, 2000a). Furthermore, in full-denture wearers, the palatal mucosa is more exposed to denture-induced trauma and this could increase the permeability of the epithelium to *Candida*, exposing epithelial ligands (*e.g.* fibronectin, laminin, collagen type I and IV) which specifically bind candidal molecules (Budtz-Jorgensen, 1990b; Cotter & Kavanagh, 2000). A correlation between *Candida*-associated denture stomatitis and type 2 DM (Odds, 1988a; Budtz-Jorgensen, 1990a) has been suggested, particularly in patients with DM-associated peripheral neuropathy. Hyperglycaemia and high salivary glucose levels can lead to a nutritionally derived enhancement of candidal growth, but they are probably not the only factors that enhance the growth of *Candida* species in the oral cavity of DM patients (Darwazeh *et al*, 1991b; Vitkov *et*

al, 1999). It is probable that the association of type 2 DM with *Candida*-associated denture stomatitis reflects some reduced immunosurveillance to *Candida.*

1.8.9.1.2 Rhinocerebral Zygomycosis (Mucormycosis)

Diabetic ketoacidosis is a major predisposing factor in rare infections caused by fungi of the family *Mucoraceae* and the class Zygomycetes (Sugar, 1992; Nussbaum & Hall, 1994; Eliopulos, 1995), indeed approximately 50% of patients with rhinocerebral murcomycosis have DM (Joshi *et al*, 1999). Usually, this infection initially arises in the nose or palate and manifests itself as a bloody ulceration of the nose or as a pseudomembranae with ulceration of the palate. Treatment requires prompt control for DM, correction of any acidosis, aggressive surgical debridement of infected tissue and systemic antifungal therapy (Scully & de Almeida, 1992).

1.8.9.1.3 Aspergillosis

Aspergillus species are filamentous saprophytes that live in soil and decaying vegetation. Aspergillosis particularly affects patients with prolonged and profound neutropenia and less frequently, invasive aspergillosis may occur in patients with DM (Rinaldi, 1983). Infections can develop in any part of the respiratory tract, including the paranasal sinuses, larynx and lungs. Primary lesions can also be localized to the eyes, ears and oral cavity (Benson-Mitchell *et al*, 1994; Myoken *et al*, 1995) and underlying structures, as well as facial skin. Oral lesions can be predominant in immunocompromised patients and are described as grey necrotic ulcers affecting the gingiva, alveolar bone, palate and occasionally the posterior of the tongue (Dreizen *et al*, 1985; Napoli & Donegan, 1991; Chambers *et al*, 1995; Myoken *et al*, 1996). Infections may spread to the brain, bones or endocardium through the bloodstream (Scully & de Almeida, 1992). Therapy for invasive

aspergillosis often requires systemic amphotericin, and systemic azoles (Vazquez & Sobel, 1995). Surgical therapy, either alone or in combination with antifungal therapy, may be required in selected patients with localised disease (Scully & de Almeida, 1992; Vazquez & Sobel, 1995).

1.8.9.2 Xerostomia

Dryness of the mouth as a feature of uncontrolled DM was first recorded in 1942 (Sheppard, 1942; Lamey *et al*, 1992). Xerostomia may be a consequence of dehydration, although long-standing oral dryness may be due to microvascular disease and neuropathy affecting the major salivary glands (Newrick *et al*, 1991). In addition, xerostomia may be result from concomitant drug therapy (antihypertensives, diuretics, anxiolytics or antidepressants) (Sharon *et al*, 1985; Albrecht *et al*, 1987; Harrison & Bowen, 1987; Finney *et al*, 1997). Prolonged xerostomia predisposes to local accumulation of plaque and debris and may contribute to the development of opportunistic oral infections (liability to dental caries, periodontal disease), altered taste (Rees, 1994; Finney *et al*, 1997), oral malodour and oral mucosal soreness.

Xerostomia in type 1 DM seems to be dependent on glucose control (Conner *et al*, 1970; Tenovuo *et al*, 1986; Sreebny *et al*, 1992; Swanljung *et al*, 1992) whereas in type 2 DM, salivary secretion seems to be influenced in particular by xerogenic drugs and autonomic neuropathy (Meurman *et al*, 1998).

1.8.9.3 Taste impairment

Diabetes mellitus can cause a loss of sweet taste sensation (Lawson *et al*, 1979; Le Floch *et al*, 1989), indeed this may be already present at time of diagnosis. Although taste impairment is usually not severe, and is generally tolerated without complaint,

the undiagnosed DM patient may favour sweet, sugary food hence exacerbating any hyperglycaemia. Many patients with polydypsia with hyperglycaemia have a predilection for sweet drinks which have a high content of refined carbohydrate (Lamey *et al*, 1992). Altered taste sensation of DM may reflect taste receptor anomalies (Hardy *et al*, 1981). In addition, sulphonylureas may cause an alteration of the taste sensation (Rollin, 1978).

1.8.9.4 Sialosis

Histologically, the enlargement of salivary glands, sometimes termed sialosis, comprises fatty infiltration of the interstitium (Davidson *et al*, 1969) and enlargement of acinar cells (Donath & Seifert, 1975). It has been previously reported that between 10% and 25% of patients with long-standing type 1 and 2 DM can develop asymptomatic, non-neoplastic enlargement of the salivary glands (Russotto, 1981; Murrah, 1985; Lamey *et al*, 1992; Greenspan, 1996), although a low correlation between DM and parotid enlargement has been recently reported: only 3% of 405 patients with DM type 1 were found to have salivary gland enlargement of this nature (Guggenheimer *et al*, 2000a). Both parotid glands are usually affected, although the submandibulary glands may be also involved (Russotto, 1981; Greenspan, 1996). It is suggested that patients may be predisposed to calculus formation and obstruction, nevertheless salivary function is largely preserved and sialosis does not influence the duration nor the severity of DM.

1.8.9.5 Dental caries

It remains unclear whether patients with DM are at increased risk of dental caries (Karjalainen *et al*, 1997; Ponte *et al*, 2001). Cross-sectional and controlled studies (Kirk & Kinirons, 1991; Jones *et al*, 1992; Swanljung *et al*, 1992) have reported

conflicting results (Reuterving *et al*, 1986; Harrison & Bowen, 1987). Several studies have suggested that poor glycaemic control could be a risk factor for caries in children and adolescents with type 1 DM (Karjalainen *et al*, 1997), while type 2 DM seemed have no effect on the prevalence of caries (Collin *et al*, 1998). Although dietary intake of carbohydrates by DM patients is lower than that of non-DM subjects, a higher number of meals per day could promote the development of caries (Lamey *et al*, 1992; Karjalainen *et al*, 1997). Any increased proneness to caries in DM may of course reflect elevated concentrations of glucose in saliva and gingival crevicular fluid of DM patients (Kjellman, 1970) but more likely, individuals with DM develop caries as a consequence of poor oral hygiene (Karjalainen *et al*, 1997).

1.8.9.6 Periodontal disease

Although not universally agreed, an increase in the risk of periodontal disease ~~association~~ with both type 1 and type 2 DM ~~has been reported~~ (Albrecht *et al*, 1987; Bacic *et al*, 1989; Emrich *et al*, 1991; Moore *et al*, 1999; American Academy of Periodontology, 2000). Uncontrolled or poorly controlled DM may give rise to increased susceptibility to oral infection, including periodontitis (Bartolucci & Parkes, 1981; Ureles, 1983) and interestingly, DM patients with severe periodontal disease are at a much greater risk of developing microvascular and macrovascular diabetic complications (Thorstensson *et al*, 1995; American Academy of Periodontology, 1999; Lalla & D'Ambrosio, 2001). The incidence of periodontitis increases among DM patients after puberty and as the adult population ages (Cohen *et al*, 1970; Cianciola *et al*, 1982; Galea *et al*, 1986; Albrecht *et al*, 1987; de Pommereau *et al*, 1992; Seppala *et al*, 1993). It has also been suggested that the disease is associated with poor glycaemic control and hyperglycaemia (American Academy of Periodontology, 2000). Epidemiological studies of Pima Indians, a group

with an extremely high prevalence of type 2 DM (Shlossman *et al*, 1990), have found that affected patients have a higher prevalence of periodontal disease than other ethnic groups, irrespective of age. Other studies (Emrich *et al*, 1991) have also found that patients with type 2 DM were more likely to have periodontal disease than non-DM control subjects, this risk not being influenced by age, gender or oral hygiene. Some studies however, have found that periodontal attachment loss, probing depth and gingivitis occur more frequently and more extensively in moderately and poorly controlled DM patients of either type than in those with good glucose control (Shlossman *et al*, 1990; Katz *et al*, 1991; Tervonen & Oliver, 1993).

However, while the facts stated above would seem to describe the most consistent trends, there is considerable conflicting data on any possible increased occurrence of periodontal disease in DM (Porter & Scully, 1994a). A number of factors could potentially contribute to any enhancement of periodontal disease in patients with DM (American Academy of Periodontology, 2000). Oral microflora, phagocytic and connective-tissue defects are the most investigated aspects in individuals affected by DM.

The function of polymorphonuclear leukocytes (PMNL) (chemotaxis, adherence, phagocytosis and killing) can be reduced in DM, leading to impaired host resistance to infection (Hill *et al*, 1974; Molenaar *et al*, 1976; Bagdade *et al*, 1978; Repine *et al*, 1980; Manouchehr-Pour *et al*, 1981; Iacono *et al*, 1985; Leeper *et al*, 1985; Wilson & Reeves, 1986; Kjersem *et al*, 1988; Marhoffer *et al*, 1992). The severity of periodontitis has been connected to defective chemotaxis; DM patients with severe periodontitis had depressed PMN chemotaxis compared to those with mild periodontitis or non-DM subjects with severe or mild periodontitis (Manouchehr-Pour *et al*, 1981; McMullen *et al*, 1981). Furthermore, decreased PMN chemotaxis has been reported in a family with a history of DM and severe periodontitis, suggesting

that the PMN defect was of genetic origin (McMullen *et al*, 1981). However, most of these PMN anomalies can usually be corrected by good glucose control.

Any increased risk of periodontal diseases in DM may be influenced by a hyperglycaemia-associated reduction in cell proliferation and growth and synthesis of collagen and glycosaminoglycans (Golub *et al*, 1978; Weringer & Arquilla, 1981; Lien *et al*, 1984; Seibold *et al*, 1985).

Table 1.1: Predisposing (local and systemic) factors of oral candidosis

(Samaranayake, 1990; Budtz-Jorgensen & Lombardi, 2000; Ruhnke, 2002)

Local predisposing factors	Systemic predisposing factors
Prostheses (changes in environmental conditions, trauma, denture usage, cleanliness)	Physiological (elderly, pregnancy, infancy)
Endogenous epithelial changes (atrophy, hyperplasia, dysplasia)	Endocrine disorders (DM, hypothyroidism, hypoparathyroidism)
Saliva-quantitative changes (xerostomia, Sjögren's syndrome, radiotherapy, drug-therapy)	Nutritional deficiency (iron, folate, vitamin B12)
Qualitative saliva changes (pH, glucose concentration)	Malignancies (acute leukaemia, agranulocytosis, others)
Commensal flora	Primary immunodeficiency (e.g. DiGeorge's syndrome)
High carbohydrate diet	Secondary immunodeficiency (e.g. HIV disease, corticosteroids, anticancer therapy)
Smoking (?)	

italics

Table 1.2: Classification of oral candidosis

(Holmstrup & Axell, 1990; Scully *et al*, 1994; Ellepola & Samaranayake, 2000a; Sitheeque & Samaranayake, 2003)

Primary oral candidosis (Group I)	Clinical features	Site involved
1. Acute		
Pseudomembranous candidosis	Semi-adherent, whitish, soft and creamy, drop-like or confluent patches. The pseudomembranes can be removed from mucosa leading a red and slightly bleeding surface.	Palate, dorsum of the tongue, buccal mucosa.
Erythematous Candidosis	Small or large erythematous areas following topical or systemic corticosteroid use, broad spectrum antibiotic therapy or in HIV disease.	Dorsum of the tongue (depapillated area), rarely palate or buccal mucosa.
2. Chronic		
Pseudomembranous candidosis	Pseudomembranous lesions may recur in patients using corticosteroids topically or by aerosol, in HIV-infected patients or in other immunocompromised patients.	Palate, oral pharynx, dorsum of the tongue.
Erythematous candidosis (*Candida*-associated denture stomatitis)	Chronic erythema and oedema of the oral mucosa in contact with a denture. The lesions are usually painless.	Palatal mucosa, the mucosa below the lower denture being rarely affected.
Chronic hyperplastic candidosis-CHC (*Candida* leukoplakia)	Chronic leukoplakia lesions infected by *Candida*. The lesions could be homogeneous or non-homogeneous (nodular, speckled). Males are more commonly affected and the lesions typically appear in long-term tobacco smokers. Histological examination of lesions is essential as some nodular CHC may exhibit epithelial dysplasia.	The lesions arise typically on the commissures of the mouth, less commonly on the buccal mucosa, palate or tongue.
Chronic multifocal oral candidosis	Rare disorders characterised by chronic candidal lesions in multiple oral sites at the same time. Most affected patients are elderly and tobacco smokers.	Multiple oral sites at the same time.
3. *Candida*-associated lesions		
Angular cheilitis	Lesions are characterised by oedema, soreness, burning and fissuring with a tendency to local bleeding. The aetiology of this infection is mainly due to the infection of *Staphylococcus aureus* and *Candida* spp.	The lesions affect the corners (angles) of the mouth.
Median rhomboid glossitis	Area of papillary atrophy, elliptical or rhomboid in shape.	The lesion is placed typically centrally in the midline of the tongue.
Secondary oral candidosis (Group II)		
	Oral manifestations of systemic mucocutaneous candidosis (thymic aplasia and candidosis endocrinopathy syndrome-CES)	

Table 1.3: Mode of action and resistance mechanism of current antifungal agents active against *Candida* spp.

(Sanglard & Bille, 2002)

Antifungal agents	Mode(s) of action	Mechanisms of resistance
Polyenes -Amphotericin B -Nystatin	Binding to ergosterol in the membrane bilayer of yeasts: loss of membrane permeability with leakage of vital cytoplasmatic components and cell death	-Absence of ergosterol or decrease of ergosterol content in cells -Increased catalase activity
Azoles **a. Imidazoles** -Ketoconazole -Miconazole -Clotrimazole **b. Triazoles** -Fluconazole -Itraconazole **c. New triazoles** -Voriconazole -Posaconazole* -Ravuconazole*	Inhibition of 14α-lanosterol demethylase (ERG11): block of 14α-demethylation step in the synthesis of ergosterol	-Enhanced efflux mediated by multidrug transporters -Decrease affinity in Erg 11p by mutations -Up regulation of ERG11 -Alteration in ergosterol biosynthetic pathway
5-FC	Inhibition of DNA/RNA synthesis: 5-FC converted (cytosine deaminase) in 5-fluroracil (5-FU) 5-FU may be converted to a nucleoside triphosphate when incorporated in RNA 5-FU may be converted to a deoxynucleoside which inhibits DNA synthesis	-Defect in cytosine permease -Deficiency or lack of enzymes implicated in the metabolism of 5-FC
Allylamines	Inhibition of squalene epoxidase: the first postsqualene enzyme of the ergosterol biosynthetic pathway, encoded by ERG1	N.R.
Echinocandins -Capsofungin	Inhibition of β-1,3 glucan synthase	-Loss of β-1,3 glucan synthase function

*Not yet available in commerce
N.R.: Not reported

Table 1.4: Typical antifungal regimens used in the treatment of oral candidosis

(Ellepola & Samaranayake, 2000a)

Drug	Form	Dosage
Amphotericin B	Lozenge, 10 mg	Slowly dissolved in mouth 3-4 X day after meals for 2 wks minimum
	Oral suspension, 100 mg/ml	Placed in mouth after meal and retained near lesions 4 times daily for 2 weeks
Nystatin	Cream	Apply to affected area 3-4 times daily
	Pastille, 100,000 units	Dissolve 1 pastille slowly after meals 4 times daily, usually 7 days Cause presence of sucrose, DM patients should avoid using this formulation of the drug
	Oral suspensions, 100,000 units	Apply after meals 4 times daily, usually 7 days and continue use for several days after post-clinical healing Cause presence of sucrose, DM patients should avoid using this formulation of the drug
Clotrimazole	Cream	Apply to affected area 2-3 times daily for 3-4 weeks
	Solution	5 ml 3-4 times daily for 2 weeks minimum
Miconazole	Oral gel	Apply to affected area 3-4 times daily
	Cream	Apply twice per day and continue for 10-14 days after the lesion heals
Ketoconazole	Tablets	200-400 mg tablets taken once or twice daily with food for 2 weeks
Fluconazole	Capsules	50-100 mg capsule once daily for 2-3 weeks
Itraconazole	Capsules	100 mg capsules daily taken immediately after meals for 2 weeks

Table 1.5: Methods of sampling the oral cavity for the isolation of *Candida* spp.

(Williams & Lewis, 2000)

Method	Procedure	Quantitative	Advantages	Disadvantages
Imprint culture	Sterile plastic foam pads of known size (typically 2.5 cm^2) placed on surface of the lesion for 30-60 min and then placed on SDA	Yes	Targets infected site. Differentiates infected and carrier state	Difficult if lesions are not evident
Oral rinse	Collection of a mouthrinse with 10 ml of sterile distilled water. After vibration, 1 ml of suspension is inoculated onto SDA	Yes	Possible detection of other microorganisms	Does not localise the infection
Swab	Gentle rubbing of a sterile cotton swab over the lesional tissue(s) and subsequent inoculation on SDA	No	Easy to use	Can remove surface epithelial layers
Whole saliva	Collection of 2 ml of saliva; solution is vibrated and 1 ml is inoculated onto SDA. Prosthesis should be removed before sampling	Yes	Good for assessing candidal carriage	May take some time
Biopsy	Biopsy specimen (excisional or incisional) is sent for histopathological examination	No	Important for diagnosis of CHC	Invasive and inappropriate for majority of *Candida* infections
Smear	Smear taken from lesional site(s), fixed onto microscope slide and stained by Gram-stain or PAS	No	Widely applicable Differentiates between yeast and hyphal form	Less sensitive

Table 1.6: Commercial test systems for the identification of *Candida* spp.
(Williams & Lewis, 2000)

System	Basis of test	Manufacturer
CHROMagar® *Candida*	Chromogenic substrate-based agar medium	Chromagar Ltd
Albicans ID	Chromogenic substrate-based agar medium	bioMérieux
Candichrom	Chromogenic substrate-based agar medium	International Microbios
Fluroplate	Fluorogenic substrate-based agar medium	Merck
API-20C AUX	Carbohydrate assimilation	bioMérieux
MiniTek	Carbohydrate assimilation	BBL Laboratories
Microring YT	Chemical sensitivity	Medical Wire & Equipment Co Ltd
Candifast	Biochemical profiling	International Microbios
RapidID yeast plus	Biochemical profiling	Innovative Diagnostics
Iatron *Candida* Check	Serology	Iatron Laboratories
AMS-YBC	Biochemical profiling	bioMérieux
Abbott MS-2	Biochemical profiling	Abbott Laboratories
Abbott Quantum II	Biochemical profiling	Abbott Laboratories

Table 1.7: Most common methods used to DNA fingerprint *Candida* isolates

(Soll, 2000; Dassanayake & Samaranayake, 2003b; Gil-Lamaignere *et al*, 2003)

Molecular techniques	How they work	Advantages and disadvantages
Restriction fragment length polymorphism without hybridisation (RFLP) or restriction enzyme analysis (REA)	It is based upon restriction endonuclease (RE) digestion of DNA at specific sites to generate shortened fragments of DNA sequences that are then separated in agarose gel. Banding patterns are based on different fragment lengths determined by the RE used.	Easy to perform and rapid. Low discriminatory power.
Restriction fragment length polymorphism with hybridisation probes	The fragments generated by RFLP can be transferred to a membrane and hybridized by Southern blot with a probe that can recognise one or more fragments of the restricted DNA. Different kinds of probe can be used for this purpose.	If the probe is carefully selected, the technique can have higher discriminatory power than conventional RFLP. Not suitable for large epidemiological studies.
Electrophoretic karyotyping and pulsed field gel electrophoresis (PFGE)	Chromosome-length polymorphism is evaluated by electrophoretic karyotype (EK) analysis, which uses electric fields of alternating orientation to move intact chromosomes through an agarose gel matrix. Larger DNA molecules (>40kb) can be separated by PFGE in which electric field is switched periodically between two different directions.	Accurate techniques but expensive equipment is necessary. Time-consuming. Karyotypes can vary as a result of phenotypic switching.
Random amplified polymorphic DNA (RAPD) or arbitrarily primed PCR	Analyse short regions of DNA sequence for variation based on the PCR priming regions. Short single primer (10-15bp) of arbitrary sequence and low stringency amplification reaction conditions are used.	Easy to perform. Due to the use of an arbitrary primer and a low annealing temperature, it is possible to encounter reproducibility problems, not only among laboratories but within a laboratory for the duration. Changes in temperature, primer-to-template-concentration ratio, could influence the presence of low-intensity bands.
Inter-repeat PCR (IR-PCR)	Based on detection of genomic regions known to be variable among eukaryotes, such as telomeric or repeated DNA. Short primers that will amplify genomic fragments flanked by inversely oriented targeted motifs.	Higher reproducibility than RAPD because the annealing temperature is higher.

Table 1.8: Chronic systemic complications of DM
(Bell & Hockaday, 1996)

Chronic systemic complications of DM	
Macroangiopathies	-artherosclerosis -myocardial disease -ischaemic heart disease -cerebrovascular disease -intermittent claudication -gangrene of feet
Macroangiopathies	-retinopathy -nephropathy -capillary basement membrane thickening
Neuropathies	-peripheral sensory neuropathy -femoral neuropathy -mononeuropathy -autonomic neuropathy a. postural hypotension b. impotence c. diabetic diarrhoea d. urinary retention e. gustatory sweating f. abnormal pupillary reflexes g. cardiac autonomic disturbance

Table 1.9: Principal techniques used to assess the glycaemic control in patients affected by DM

(Viberti *et al*, 1979; Parving *et al*, 1982; Viberti *et al*, 1982; Pickup *et al*, 1984; Lugari *et al*, 1988; Bell & Hockaday, 1996; Goldstein *et al*, 2003)

Principal techniques used to assess glycaemic control in DM	Advantages	Disadvantages
Blood glucose monitoring	Self-monitoring Results available immediately Avoids ketoacidosis and encourages good patient cooperation	Not precise measure of glycaemic control Not diagnostic
Glycosylated haemoglobin (HbA_{1c})	Accurate and objective measure of glycaemic control over past weeks 2-3 months	HbA_{1c} may be incorrectly reduced in anaemia or during pregnancy
Plasma fructosamine	Good if HbA_{1c} cannot be measured (e.g. haemolytic anaemias)	It provides an index of glycaemic status over the preceding 1-2 weeks only
Urine testing	Useful for monitoring the levels of urinary proteins and ketones of renal insufficiency in DM patients	Usually not good enough as a diagnostic and control test

Table 1.10: Major hypoglycaemic agents used to control DM type 2

Drug class	Daily dose Range (mg)	N° of daily doses	Site of metabolism	Mechanism of action
Sulphonylureas				**Stimulate insulin secretion**
Glibenclamide	5-15	1-3	Oxidised in liver-excreted urines; 50% excreted unchanged in faeces	
Gliclazide	40-320	1-3	95% liver	
Glimepiride	1-4/6	1		
Glipizide	2.5-20	2-3	90-95% liver-excreted unchanged in urine; 12% excreted in faeces	
Gliquidone	15-180	2-3	90-95% liver metabolites excreted in bile	
Biguanides				**Increase hepatic glucose output**
Metformin	500-1700	1-3	Liver, not metabolized. Excreted unchanged in urine	
Alpha-Glucosidase inhibitors				**Decrease gastro-intestinal absorption of carbohydrates**
Acarbose	25-100	1-3	Liver	
Miglitol			Liver	
Meglitinides				**Stimulate insulin release**
Repaglinide	0.5-1/16	1-3	Mostly excreted in urine	
Thiazolidine-diones				**Reduce peripheral insulin resistance**
Pioglitazone	15-30	1	Liver	
Rosiglitazone	4-8	1-2	Liver	
Troglitazone	400-600	1	Liver (liver toxicity)	WITHDRAWN

Table 1.11: Principal insulin preparations used to control DM

Insulin preparation types	Onset of duration (h-min)	Peak of action (h)	Maximal duration of action (h)	Commonly used regimens
Short-acting insulin	0.5-1 h	1-3	6-8	Given 20-30 min before meals
Insulin Soluble	30 min	1-3	8	
Rapid-acting insulin analogue	10-30 min	1-3	3-5	Given immediately before meals
Insulin Lispro	30 min	1-2	8	
Insulin Aspart	30 min	1-2	8	
Intermediate-acting insulin	1.5-2 h	4-12	18-24	1. Given before bed 2. Given before bed, in combination with tablets for overweight patients 3. Given once daily before breakfast, for elderly 4. Given twice a day, for type 2 DM
Long-acting insulin	1-2 h	4-12	20-40	Once daily, or twice daily with a short-acting (soluble) insulin
Biphasic action (combination of rapid or short and intermediate action)	0.5-1 h	1-10	18-24	
10/90 (10% soluble-90% isophane insulin)	30 min	2-8	24	Given 20-30 min before breakfast and evening meal, as a twice daily regimen
20/80 (20% soluble-80% isophane insulin)	30 min	2-8	24	Given 20-30 min before breakfast and evening meal, as a twice daily regimen
30/70 (30% soluble-70% isophane insulin)	30 min	2-8	24	Given 20-30 min before breakfast and evening meal, as a twice daily regimen
40/60 (40% soluble-60% isophane insulin)	30 min	2-8	24	Given 20-30 min before breakfast and evening meal, as a twice daily regimen
50/50 (50% soluble-50% isophane insulin)	30 min	2-8	24	Given 20-30 min before breakfast and evening meal, as a twice daily regimen
25% insulin Lispro – 75% insulin Lispro Protamide	30 min	2	24	Given immediately before breakfast and evening meal, as a twice daily regimen

Table 1.12: Oral *Candida* carriage in patients with DM investigated by different authors

1. Authors	2. Oral *Candida* carriage in DM patients(%)	3. Sampling methods	4. Total number of DM patients investigated	5. Number of patients with DM type 1	6. Number of patients with DM type 2	7. Oral *Candida* carriage in controls (%)	8. Increased oral *Candida* carriage in DM patients denture wearers	9. Oral *Candida* carriage related to type of DM	10. Increased oral *Candida* carriage with poor metabolic control
Barlow *et al*, 1969	62.5%	Swab	24	----**	----**	35%	Not investigated	Not investigated	No
Tapper-Jones *et al*, 1981	60%	Imprint culture	50	----**	----**	42%	Yes	Not investigated	Not investigated
Fisher *et al*, 1987	51%	Oral rinse	412	232	180	-	Yes	Not investigated	No
Lamey *et al*, 1988	57%	Oral rinse	106	57	52	27%	Yes	No difference	No
Hill *et al*, 1989	49%	Swab	51	----**	----**	-	Yes	Not investigated	Yes
Darwazeh *et al*, 1990	54%	Swab and smear	50	19	31	40%	No	No difference	No
Aly *et al*, 1992	66%	Swab and oral rinse	436	231	205	-	No	Yes: more *Candida* carriers in DM type 1	No (oral rinse), yes (palatal carriage in dentures)
Dorocka-Bobkowska *et al*, 1996	54%	Swab and imprint culture	70	0	70	41%	Yes	Not investigated	No
Willis *et al*, 2000b	77%	Oral rinse	414	414	0	-	Not investigated	Not investigated	No
Guggenheimer *et al*, 2000a	23%	Cytologic smear	405	405	0	5.7%	Yes	Not investigated	Yes
Manfredi *et al*, 2002	60%	Oral rinse	137	56	83	57%	Yes	No difference	No

1. Studies that have investigated the oral *Candida* carriage in patients affected by DM
2. Oral *Candida* carriage (cfu/ml) in DM patients is expressed in percentage
3. Methods of sampling that have been used to evaluate the oral *Candida* carriage in the studies
4. Number of DM patients investigated in the studies
5. Number of patients affected by DM type 1 investigated in the studies
6. Number of patients affected by DM type 2 investigated in the studies
7. Oral *Candida* carriage (cfu/ml) in controls is expressed in percentage
8. Difference of oral *Candida* carriage (increased levels of cfu/ml) in DM patients wearing a denture in comparison to non denture wearers.
9. Difference of oral *Candida* carriage (cfu/ml) in patients affected by type 1 DM in comparison to patients affected by type 2 DM
10. Difference of oral *Candida* carriage (cfu/ml) in DM patients with poor metabolic control in comparison to DM patients with good metabolic control.

**: In these studies, the researchers did not differentiate between type 1 DM and type 2 DM

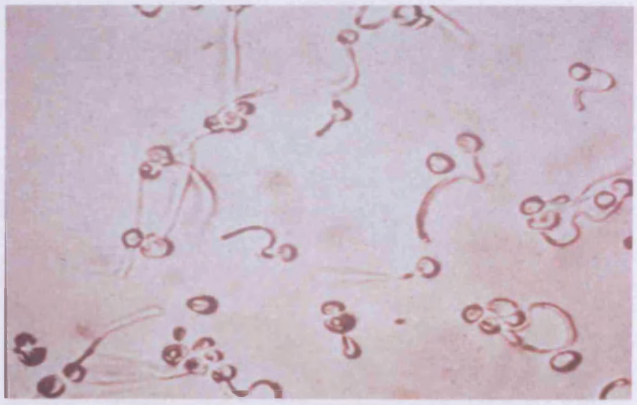

Figure 1.1: ***In vitro C. albicans*** **germ-tube**

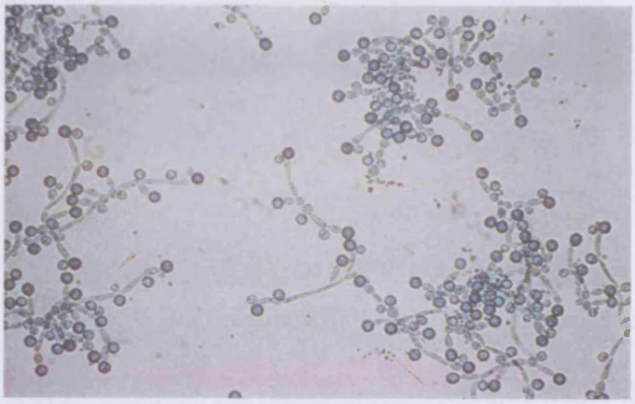

Figure 1.2: ***In vitro C. albicans*** **clamydospore**

Figure 1.3: Pseudomembranous candidosis of the palate

Figure 1.4: Acute erythematous candidosis

Figure 1.5: Chronic hyperplastic candidosis (candidal leukoplakia)

Figure 1.6: Denture stomatitis associated with *Candida*

Figure 1.7: Unilateral angular cheilitis associated with *C. albicans*

Figure 1.8: Bilateral angular cheilitis associated with *Candida* in an elderly woman affected by DM type 2

Figure 1.9-1.10: Median rhomboid glossitis

The lesion is associated with the presence of *C. albicans* (left) and kissing lesion of the plate in the same patient (right)

1.9 Summary

The range of human infections caused by *Candida* spp. is considerable. They vary from local conditions, such as oral and general thrush, to fatal infections in patients who are already seriously ill with other diseases.

Candida spp. have been frequently isolated from the oral cavity of patients affected by DM (Darwazeh *et al*, 1990; Aly *et al*, 1995; Willis *et al*, 1999). Much has been written about the incidence of the oral carriage of *Candida* spp. and candidal infections in the mouth of patients with DM compared with non-DM control subjects (Dorocka-Bobkowska *et al*, 1996; Abu-Elteen & Abu-Alteen, 1998; Willis *et al*, 1999; Willis *et al*, 2000b; Willis *et al*, 2001). However, controversy still exists as to whether the carriage of *Candida* spp. and the oral manifestations of candidal infections are more common in the oral cavity of DM patients than in non-DM individuals and whether this is related to oral and/or systemic factors.

Furthermore, the epidemiology of *Candida* spp. has changed over recent years, with selective pressure from increased use of antifungals. Differences in geographic distribution and patient populations of *Candida* spp. and *C. albicans* genotype have also been reported in several studies (McCullough *et al*, 1999b).

1.10 Aims of the thesis

The aims of this study were:

1. To assess the prevalence of *Candida* carriage in the oral cavities of two groups of patients with DM and of a group of healthy non-DM subjects;
2. To genotype the *Candida* isolates from the studied populations;
3. To evaluate extracellular proteinase production *in vitro* and the ability to adhere *in vitro* to fibronectin of the oral *Candida* isolates from the studied groups;

4. To assess the *in vitro* antifungal susceptibility of the *Candida* isolates from the oral cavities of the patients affected by DM and from the control group;
5. To assess the phylogenetic relatedness between *C. albicans* strains isolated from two groups of patients with DM and from the non-DM control subjects;
6. To evaluate the *in vitro* activity of a monoclonal antibody (mAbK10) and of a synthetic decapeptide (KP) on the *Candida* isolates of the oral cavities of DM patients and non-DM subjects.

1.11 Hypotheses of the thesis

Due to the altered oral environment of DM patients compared with healthy control subjects, it is hypothesised that there is an associated variation in the prevalence and type of *Candida* spp. that colonise the oral cavity of patients with DM. The following phenotypic and genotypic features of *Candida* were characterised to test this hypothesis:

1. Variation in the frequency of *Candida* spp. isolation, degree of oral colonisation, *Candida* species and *C. albicans* genotypic distribution of oral yeasts isolated from patients with DM and a non-DM control group;
2. Differences in the *in vitro* ability to produce extracellular proteinases and to adhere to fibronectin of *Candida* isolates from patients with DM and non-DM individuals;
3. Differences in the *in vitro* antifungal susceptibility to six different antifungal agents of *Candida* isolates from the oral cavity of patients with DM and from healthy subjects;
4. Variation in the genetic relatedness of *C. albicans* isolates of patients with DM from two different geographic locales and from a group of non-DM subjects;

5. Differences in the *in vitro* candidacidal activity of mAbK10 and of KP against isolates of different *Candida* spp. and with a different susceptibility to conventional antifungal agents isolated from patients with DM and from healthy individuals.

CHAPTER 2

Patients and General Methods

2.1 Patient samples

2.1.1 UK patients with DM

Ethical approval for the project was granted by the Joint Ethics Committee of the University College of London, University College London Hospitals (UK). All patients gave written informed consent prior to being enrolled in the present study.

One hundred and forty-two patients attending an outpatient Diabetology Clinic at Middlesex Hospital, UCLH, London, were enrolled during their routine diabetic review appointment. The medical history of each patient was recorded at the time of examination. This included tobacco smoking habits, type of DM (type 1 or type 2) and duration of the disease (time since diagnosis <10 years; >10 years). The type of DM was diagnosed by a specialist diabetologist using the guidelines of the American and British Association of Diabetes (British Diabetic Association, 1997; The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998; American Academy of Periodontology, 2000). The presence of the most common long-term complications of DM (retinopathy, nephropathy and peripheral neuropathy) was similarly recorded. The diagnosis of these conditions was based on clinical reports provided by ophthalmologists, nephrologists and neurologists attending each patient.

Glycaemic control, assessed by HbA_{1c} (glycosylated haemoglobin), was used as a measure of recent diabetic control. Patients were classified into three mutually

exclusive groups, depending on the level of haemoglobin glycosylation at the time of oral examination (group 1: <7.5%; group 2: >7.5%, <8.5%; group 3: >8.5%).

All patients received a detailed oral examination and oral rinses (Section 2.2) were used to assess the growth and the degree of yeast colonisation for each patient. Patients were asked to rinse their mouth with 10 ml of sterile distilled water for 1 min and expectorate back into a 50 ml sterile container.

2.1.2 Italian patients with DM

Ethical approval for the project was granted by the Parma Hospital Medical Committee on the Ethics of Human Research. All patients gave written informed consent prior to being enrolled in the present study. A medical history, detailed oral examination and estimation of yeast carriage by oral rinse was obtained as was the case for the UK DM patients above. In total 107 patients attending an outpatient Diabetology Clinic at the Parma Hospital, Parma, Italy, were enrolled during their routine review appointments for their DM.

2.1.3 Patients without DM

A total of 130 healthy, non-DM subjects were enrolled in London, UK. Patients were attending either a General Dental Clinic (n = 41), a Special Needs Clinic (n = 42), a Prosthodontic Clinic (n = 31) or an Oral Medicine Clinic (n = 16). The medical history of each patient was recorded, including current medication and tobacco smoking habits. All patients received a detailed oral examination, which also recorded the presence or absence of dentures (either partial or full) and the presence of any mucosal abnormalities. As with the DM patients oral rinses were obtained to determine the presence of yeast in the oral cavity.

2.2 Concentrated oral rinse

Oral rinse samples were assessed for qualitative/quantitative estimation of yeast colonisation. Mouth swills (30 s) using 10 ml of sterile distilled water, were collected from each patient in a 50 ml sterile tube. Each mouth rinse was vortexed for 30 s and 100µl were spread evenly on a SDA (Sigma-Aldrich, Dorset, UK) plate containing chloramphenicol (1mg/l, Sigma-Aldrich). Furthermore, two 10-fold dilutions (in sterile distilled water) of the vortexed mouth rinse were plated onto SDA/chloramphenicol plates for enumeration of the degree of colonisation. Each plate was incubated for 48 h at 37°C.

2.3 Cfu/ml determination

Growth of *Candida* was quantitatively assessed by enumeration of cfu per ml of mouth rinse. Non-identical, single colonies were isolated and cultured (re-incubated for 48 h at 37°C) to confirm their identity.

2.4 Species determination

2.4.1 Phenotypic methods for identifying *Candida* species

Each representative colony type from the oral sample was incubated in 1 ml of horse serum (Sigma-Aldrich) for 2 to 4 h at 37°C. Approximately 20 µl was placed on a glass slide and microscopically examined to identify whether germ-tubes were present. The presence of germ-tubes gave a positive identification for *C. albicans* and *C. dubliniensis*. Each strain was subsequently stored in Lennox L Broth Base (2g of Lennox L Broth Base-Sigma Aldrich, 50 ml of sterile water and 50 ml of glycerol, Sigma-Aldrich) in a 2 ml Eppendorf tube and kept at –20°C for subsequent analysis.

2.4.2. Genetic identification of isolates at species level

Genomic DNA was extracted by spheroplast formation and precipitation in ethanol as detailed elsewhere (Scherer & Stevens, 1987) over a three day time period.

Each strain was cultured on a SDA plate for 24 h at 37°C, inoculated in 1 ml of 1 M sorbitol pH 7.5 (Sigma-Aldrich) and then vortexed. After centrifugation at 5000 rpm (2700 g) (Centrifuge 5417C, Eppendorf, Hamburg, Germany) for 10 min, the supernatant was removed and re-suspended in 1 ml of spheroplast buffer. This spheroplast buffer consisted of 42 µl 2-mercaptoethanol (0.1% v/v) (Sigma-Aldrich), 0.2 µg zymolase (Sigma-Aldrich), 2 ml of phosphate buffer 50 mM (Sigma-Aldrich) and 40 ml 1M sorbitol pH 7.5 (Sigma-Aldrich). The cells were incubated overnight at room temperature to allow the spheroplasts to form. The supernatant was removed after centrifugation at 5000 rpm (Centrifuge 5417C) for 10 min and re-suspended in 1 ml of lysis buffer consisting of 50 mM EDTA (Sigma-Aldrich) and 2% sodium dodecyl sulphate (SDS) (Sigma-Aldrich), pH 8.5. Spheroplasts were incubated for 1 h between 65°C and 70°C. The lysed cells were allowed to cool to room temperature and 50 µl of 5M potassium acetate (Sigma-Aldrich) were added. Subsequently, each sample was incubated for 1 h on grounded ice to allow for complete precipitation of the SDS and protein. A 0.5 ml volume of supernatant was carefully removed and added to 1ml of 100% ethanol (Sigma-Aldrich) to allow the nucleic acid to precipitate. Precipitated nucleic acids were collected by centrifugation at 11000 rpm (Centrifuge 5417C) for 10 min and the resultant pellet allowed to air dry. The dried pellet was re-suspended in 0.5 ml of a Tris-EDTA buffer (10mM Tris and 1M EDTA, pH 7.5 supplied in ready-mix pellets by Bio-Rad, Marnes La Coquette, France) containing 1µg of ribonuclease A (1.4 x 10 to 4 w/v) (Sigma-Aldrich) and incubated overnight at room temperature.

DNA was precipitated by the addition of 100% isopropanol (Sigma-Aldrich) in a 1:1 ratio and centrifuged at 11000 rpm (Centrifuge 5417C) for 10 min. The supernatant was discarded, the resultant pellet was re-suspended in 1 ml of 75% ethanol to remove cations and the DNA was finally collected by centrifugation at 11000 rpm (Centrifuge 5417C) for 2 min. The DNA pellet was air dried and suspended in 200 µl of TE (Sigma-Aldrich). Each sample was stored at 4°C and diluted 1:10 for use in the PCR.

2.4.2.1 PCR for species identification

PCR amplifications were performed on DNA preparations from all yeast isolates to identify the *Candida* species. PCR primers were directed against the V3 region of the 25S rDNA, and the intergenic transcribed spacer region (ITS) of rDNA.

Primers for PCR were designed for two separate areas of the DNA, encoding the RNA. The first pair of primers, ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC -3'), has been used in previous studies (Williams *et al*, 1995; Nho *et al*, 1997; Gilfillan *et al*, 1998; McCullough *et al*, 1999b; McCullough *et al*, 1999a). These give an expected PCR product extending from the 5' end of the 18S rDNA to the 3' end of the 25S rDNA and include both the ITS regions (ITS1 and ITS2) as well as the entire 5.8S rDNA.

The second pair of primers, CA25SV3-L (5'-TCT TAA CAG CTT ATC ACC CTG GAA TTG GTT-3') and CA25SV3-R (5'-ATT GTG TCA ACA TCA CTT TCT GAC CAT CAC-3'), was designed from sequences submitted to Gen Bank (McCullough *et al*, 1999b; McCullough *et al*, 1999a) and produces a PCR product that spans the V3 region of the 25S rDNA (Sullivan *et al*, 1995).

The DNA of the isolates was amplified in a reaction volume of 20µl containing 1µM from each primer (0.5µl), 0.5 U (0.5µl) of Red *Taq* polymerase (Sigma-Aldrich) and

Red *Taq* buffer (supplied by the same manufacturer as a 10× concentrate and used to give a final concentration of 1.1 mM $MgCl_2$, 0.2 mM of dATP, 0.2 mM of dCTP, 0.2 mM of GTP and 0.2mM of dTTP.

The reactions were performed with an automated heated-lid thermal cycler (Techne, Genius) by incubation for 3 min at 94°C before 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 2.5 min prior to a final elongation step of 72°C for 10 min.

The amplicons of the V3-25S and ITS PCR were digested with restriction enzymes *Hae* III and *Dde* I, respectively. Endonuclease digestion was achieved by overnight incubation (to allow complete digestion of the PCR amplimers) with 10 U of enzyme and with the corresponding buffer at 37°C. It has been shown that this method discriminates between all medically important species of *Candida* (McCullough *et al*, 1999b; McCullough *et al*, 1999a).

Ten microliters of the PCR products, with and without endonuclease digestion, were analysed by electrophoresis in a 2% agarose gel in TAE (Sigma-Aldrich) (40 mM of Tris acetate, pH 8.3, containing 1 mM of EDTA) buffer. Bands were visualised by UV transillumination at 302 nm after ethidium bromide staining.

2.5 Defining isolates at sub-species level using genotypic methods

2.5.1 Broad sub-species groups via the presence and size of rDNA intron.

The primers used in this assay for the PCR were CA-INT-L (5'-ATA AGG GAA GTC GGC AAA ATA GAT CCG TAA-3') and CA-INT-R (5'-CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-3'). These primers were directed against the transposable intron in the 25S rDNA as previously described (Mercure *et al*, 1993; McCullough *et al*, 1999b; McCullough *et al*, 1999a).

PCR reaction mixes were used for species identification as described previously (Section 2.4.2.1) except that CA-INT-L / CA-INT-R primers were used. The cycling parameters were identical to those described for species identification.

Ten microliters of the PCR products were analysed by electrophoresis as described previously (Section 2.4.2.1).

Candida albicans isolates were thus divided genotypically into sub-groups A, B, or C depending on the presence or absence of this 25S rDNA transposable intron as described previously (Scherer & Stevens, 1987; Stevens *et al*, 1990; Clemons *et al*, 1991; Clemons *et al*, 1997; McCullough *et al*, 1999b; McCullough *et al*, 1999a).

Using the primers described above, it was expected that a single PCR product of ~ 450 bp and of ~ 840 bp would be obtained for *C. albicans* genotypes A and B, respectively. The *C. albicans* genotype C isolates were expected to yield two PCR products (C: ~ 450 bp and ~840 bp) that were identical in size to the respective products from *C. albicans* genotype A and B. All *C. albicans* genotype D isolates would be expected to yield a PCR product identical in size to that of *C. dubliniensis* (~1080 bp) (McCullough *et al*, 1999b).

2.5.2 Identification of isolates at sub-species level by PCR fingerprinting

PCR fingerprinting was performed on *C. albicans* samples isolated from each group of patients to evaluate the genetic relationship between the groups of isolates.

The DNA from randomly selected *C. albicans* isolates from DM patients in Parma (n = 30), DM patients in London (n = 31) and London control patients (n = 30) was assessed by PCR fingerprinting with 5 different short random primers (Xu *et al*, 2000):

M13: 5^1- GAC GGT GGC GGT TCT-3^1

T3B: 5^1 - AGG TCG CGG GTT CGA ATC-3^1

TELO 5^1 – TGG GTG TGT GGG TGT GTG GGT GTG-3^1

$(GACA)_4$: 5^1- GAC AGA CAG ACA GAC A-3^1

OPA-03: 5^1- AGT CAG CCA C-3^1

The DNA of the isolates and a reaction mix identical to those previously described (Section 2.4.2.1) was amplified in a reaction volume of 20µl containing 1µM primer (0.5µl), 0.5 U (0.5µl) of Red *Taq* polymerase (Sigma-Aldrich) in Red *Taq* buffer (Section 2.4.2.1).

PCR fingerprinting with M13, T3B, TELO and $(GACA)_4$ was performed with a heated-lid thermocycle PCR machine (Techne, Genius) for 1 cycle at 97°C for 3 min, 40 cycles for 40 s at 93°C, 2 min at 50°C, 40 s at 72°C and a final step of 10 min at 72°C.

PCR fingerprinting with OPA-03, which requires lower temperatures, was performed for 1 cycle at 97°C for 3 min, 45 cycles for 1 min at 93°C, 2 min at 36°C, 2 min at 72°C and a final step of 10 min at 72°C, as previously reported (Xu *et al*, 2000).

Ten microliters of the PCR products were analysed by electrophoresis through a 2% w/v agarose gel (SeaKem Gold, FMC BioProducts, Rockland, ME, USA) in TAE buffer (40mM of Tris-acetate, Sigma-Aldrich and 0.2 mM of EDTA, Sigma-Aldrich; pH 8.3) for 2 h at V/cm and visualised by UV transillumination following ethidium bromide staining. This resulted in each isolate having 5 distinct PCR fingerprints, one for each of the primers, for their identification at sub-species level.

2.6 Adhesion assay

The ability of individual isolates to adhere to paramagnetic beads coated with fibronectin was assessed in an *in vitro* assay. A single colony from a 48 h, 37°C SDA culture was inoculated in 5 ml of Yeast, Peptone, and Dextrose (YPD) broth. This broth consisted of 20 g of dextrose powder (Sigma-Aldrich), 20 g of peptone powder (Sigma-Aldrich) and 10 g of yeast extract (Sigma-Aldrich) in 1L of water. This inoculum was incubated for 18 h at 37°C, after which 45 ml of YPD was added and incubation continued for a further 6 h at 37°C. The cells were then centrifuged at 3600 rpm (2753 g) (Centrifuge TJ-25 Beckman Coultur, Palo Alto, California, USA) for 3 min and washed three times in TE buffer pH 8 (1X: 10mM Tris HCl pH 8, 1 mM EDTA pH 8; Sigma-Aldrich) to remove traces of YPD. The cell pellet was then re-suspended in 5 ml of TE buffer pH 7.8. The number of yeasts present was counted with a haemocytometer and adjusted to 10^8 cells/ml.

In vitro adhesion was assessed using paramagnetic beads (Dynabeads® M-450 Tosylactivated, Dynal Biotech UK, Wirral, UK) coated with fibronectin (0.1%, Sigma-Aldrich). Non-specific sites were blocked with bovine serum albumin (BSA, Sigma-Aldrich) as outlined by the manufacturer (Dynal Biotech UK).

To coat the paramagnetic beads (Dynal Biotech UK) with fibronectin, they were initially vortexed in a 15 ml tube, which was then placed in the associated magnet for 2 min. The supernatant was then discarded, the tube removed from the magnet and 1ml of Buffer A (0.019 M NaH_2PO_4 and 0.081 M Na_2HPO_4, at pH 7.4) was added. The washing procedure was repeated twice.

Fibronectin 50 µg (Sigma-Aldrich) was added to the suspension which was incubated for 10 min at 37°C on slow tilt. Ten µl of Buffer B was added (80 ml of Buffer B

consisted of 0.88 g of NaCl, 0.1 g of BSA and ten-fold diluted Buffer A diluted in distilled water) and the suspension was incubated at slow tilting for a further 24 h at 37°C. The mixture was put in the magnet for 3 min and the supernatant was carefully removed. One millilitre of cold Buffer B was added and the suspension was cooled in the fridge for 5 min. This procedure was repeated twice and 1 ml of Buffer C (100 ml contained 2.42 g of Tris, Sigma-Aldrich, and 0.1 g of BSA, pH 8.5) was added. The suspension was incubated at 37°C for 4-6 h. The mixture was placed in the magnet for 3 min and the supernatant was carefully removed. One millilitre of cold buffer B was added and the suspension was cooled in the fridge for 5 min.

Dynabeads coated primarily with fibronectin and secondarily with BSA were then suspended in Buffer B and the concentration adjusted to 10^8 beads/ml (stock solution) and finally stored at 4°C for subsequent use. A final 100-fold dilution of the stock beads (990 µl of Buffer B with 10 µl of beads stock solution) was prepared and used in the assay (10^6beads/ml).

The Dynabeads coated with fibronectin were incubated with the yeast cells in a 200 ml final volume in the TE buffer pH 8 (Sigma-Aldrich). The mixture was incubated at a ratio of 10^6 beads/ml dynabeads to 10^8cells/ml yeast cells and shaken for 30 min at room temperature. The cells bound to the Dynabeads were collected for 3 min using the magnetic separator supplied (Dynal MPC, Dynal Biotech UK). Unbound yeast cells were removed from the solution by washing three times with TE buffer. The adherent yeast cells were finally disassociated from the Dynabeads by re-suspending them in 0.1 N NaOH (150 µl). Free cells and Dynabeads were counted using a haemocytometer and results were expressed as number of *Candida* cells bound per fibronectin-coated Dynabead.

2.7 Extracellular proteinase production

The ability of the individual isolates to produce extra-cellular proteinases was assessed in an *in vitro* assay as previously described (Macdonald & Odds, 1980). A culture medium containing 1000 ml of sterile water, 2g of BSA (Sigma-Aldrich), 20 g of dextrose (Sigma-Aldrich), 1g of KH_2PO_4 (Sigma-Aldrich), 0.5 g of $MgSO_4$ (Sigma-Aldrich) and a synthetic vitamin solution (20 µg of biotin, 200 µg nicotinic acid, 200 µg of riboflavin, 400 µg of thiamine and 400 µg of pyridoxal hydrocloride, Sigma-Aldrich) was prepared. A single colony from an 18 h, 37°C SDA culture of each isolate was inoculated in 1 ml of the medium and was shaken for 5 days at room temperature. The number of yeasts per ml was enumerated using a haemocytometer and then removed by centrifugation at 1500 rpm (Centrifuge TJ-25) for 30 min; 0.5 ml of the supernatant was incubated in 2 ml of 10g/L BSA solution (Sigma-Aldrich) in 0.05 M citric buffer (as protein substrate) (Sigma-Aldrich) pH 3.2, for 30 min at 37°C. The reaction was stopped by adding 5 ml of trichloracetic acid solution on ice (TCA, 50 g/L, Sigma-Aldrich) for 15 min and the precipitate was removed by centrifugation. In negative control samples, incubation with BSA and citric buffer was omitted. The absorbance of each sample at 280 nm was determined against a citrate-buffer blank and representative fractions containing ultraviolet-absorbing material were assayed for proteinase activity. Results were expressed as a ratio between the spectrophotometer reading and numbers of cells/ml.

2.8 *In vitro* antifungal susceptibility

The susceptibility of the individual isolates to commercially available antifungal agents was assessed by a broth microdilution assay using alamar blue in the Bio-Rad *in vitro* assay, Fungitest. A single colony from a 48 h, 37°C SDA culture of each isolate was diluted into 3 ml of distilled water to give a turbidity reading of

McFarland number 1 (equal to approximately 3 x 10^6 cells/ml). One hundred µl of this suspension was diluted into 1.9 ml of sterile distilled water and 20 µl was added to 3 ml of a suspension medium (supplied by Bio-Rad). One hundred µl (approximately 1 x 10^3 cells/ml) of the yeast suspension ~~were~~ was added to each of the 16 wells of a Fungitest® microplate (Bio-Rad). This 16-well microplate contained six different antifungal agents at two different concentrations (fluconazole 8 and 64 µg/ml, itraconazole 0.5 and 4 µg/ml, miconazole 0.5 and 8 µg/ml, ketoconazole 0.5 and 4 µg/ml, amphotericin B 2 and 8 µg/ml and 5-FC 2 and 32 µg/ml), in modified RPMI 1640 buffered medium, with the presence of a redox indicator (alamar blue). Two growth control wells and 2 negative control wells were also present in each microplate.

After incubation at 37°C for 48 h, growth was assessed by colorimetric means as outlined by the manufacturer. Results were expressed as resistant, intermediately resistant or susceptible to each of the antifungal agents tested.

2.9 *In vitro* evaluation of the candidacidal activity of the monoclonal anti-idiotypic antibody K10

Anti-idiotypic antibodies representing the internal image of PaKT and able to mimic its biological activity have been recently obtained (Section 1.7). Such antibodies have been produced in monoclonal (KTmAb; K10) and recombinant format (KTscFv) by idiotypic vaccination using a PaKT-neutralising monoclonal antibody (mAbKT4) as an immunogen (Magliani *et al*, 1997a; Polonelli *et al*, 1997) (Section 8.1).

2.9.1 KT-IdAb (K10) purification

Hybridoma cells secreting K10 (Polonelli *et al*, 1997) were grown in RPMI 1640 (Sigma-Aldrich) medium supplemented with L-glutamine (0.3 g/l, Sigma-Aldrich),

pyruvic acid (12 mM, Sigma-Aldrich), non-essential amino acids (Mem non-essential amino acid solution 100X, Sigma-Aldrich), vitamins (Mem vitamin solution 100X, Sigma), penicillin-streptomycin solution (streptomycin 15 mg/100 ml, penicillin 15,000 U/100 ml, Sigma), and 15% foetal bovine serum (Sigma-Aldrich). Supernatant from the culture of these hybridoma cells was collected, precipitated in saturated ammonium sulphate (38.35 g $(NH_4)_2SO_4$ in 50 ml of distilled water at 25°C overnight and re-suspended in KH_2PO_4 0.01M pH 7.44) solution (1:1, Sigma-Aldrich) and then dialyzed against phosphate buffered saline (PBS, Sigma-Aldrich) for 48 h. After dialysis, the purified suspension was filtered through a 0.22 µm filter (Millex-GP, Millipore, Bedford, USA). Antibody concentration was determined by capture ELISA using a pair of mouse monoclonal antibodies against a µ heavy chain of rat Ig (LO-IMEX, Brussels, Belgium). In brief, the wells of a polystyrene MicroElisa plate (Costar, Corning Incorporated, Corning, New York, USA) were coated overnight at 4°C with 100 µl of a purified MAb (MARM-7, a mouse IgG1 against a µ heavy chain of rat Ig, Technopharm, Paris, France) at a concentration of 5 µg/ml in 0.05 M sodium carbonate/bicarbonate buffer (10X: 15.9 g of $NaCO_3$, 29.3 $NaHCO_3$ dissolved in sterile water, final volume 1 L) at pH 9.6. The wells were then washed 5 times with PBS (Sigma-Aldrich), Tween 20 (0.1%) (Sigma-Aldrich) using Ascent Well-Wash (Lab-Systems Oy, Helsinki, Finland) and saturated for 1 h at 37°C with 2% skimmed milk (Sigma-Aldrich) dissolved in PBS. After a second cycle of washing 5 times, as described above, 100 µl of pre-determined scalar dilutions (100, 50, 25, 12.5 ng/ml) of a standard rat IgM MAb (LOMA 7, Technopharm) was added to a plate in four pairs of wells. One hundred µl of pre-determined scalar dilutions (1:1000-1:8000) of the rat MAb (K10) for testing was added to second group of four well pairs on the same plate. The suspensions were incubated for 1 h at 37°C. The washing procedure described above was repeated again 5 times. One hundred µl of a

mouse MAb labelled with peroxidase (MARM4-peroxidase, a mouse IgG1 against μ heavy chain of rat Ig, Technopharm) diluted 1:2000 in PBS was added to each well on the plate. The plate was incubated for 1 h at 37°C. The washing procedure described above was repeated again 5 times. Peroxidase activity was detected using a 3,3'-5,5' tetramethylbenzidine dihydrochloride tablet as a substrate (Sigma-Aldrich), dissolved in 10 ml of 0.05M phosphate-citrate buffer (Sigma-Aldrich) pH 5 supplemented with 2 μl of fresh 30% hydrogen peroxide (Sigma-Aldrich). Reactions were stopped after 15 min at room temperature by adding 50 μl of 2M H_2SO_4 to each well. Optical density (OD) of the reaction mixes was recorded at 450 nm using an Ascent Multiskan spectrophotometer (Labsystems Oy, Helsinki, Finland).

A standard curve was constructed (GraphPad, Prism 4, GraphPad Software, Inc., San Diego, USA) by plotting the standard scalar concentrations of rat IgM Mab LOMA-7 (on the X-axis) against the acquired corresponding OD values (on the Y-axis). The unknown value of K10 concentration was obtained by interpolating the values on the standard curve (GraphPad Software).

2.9.2 Candidacidal activity of the monoclonal antibody K10

The candidacidal activity of the anti idiotypic antibody K10 was evaluated *in vitro* by a cfu assay on randomly selected *Candida* strains from each patient group (23 strains from DM patients from Parma, 24 from DM patients from London and 24 strains from controls) (Chapter 8, Table 8.1). Strains were grown on SDA at 30°C for 24 h. The yeast cells from a single colony were suspended in M199 medium (Sigma-Aldrich) and incubated at 37°C while shaken for 2 h. *Candida* cells were counted using a haemocytometer and 10 μl of yeast cell suspension (2-3 × 10^2 cells) was added to 90 μl of K10 (final concentration 20 μg/ml). Previously heat-inactivated K10, also at the same concentration, was used as a control. A reference strain of *C.*

albicans (10S), known to be sensitive to K10 (Conti *et al*, 1996; Magliani *et al*, 1997a), was used as a positive control. Following 18 h incubation at 37°C, the suspensions were dispensed onto SDA plates, and incubated at 30°C for 48 h before cfu enumeration. Each experiment was performed in triplicate and the results expressed as a percentage of inhibition of each isolate against the heat-inactivated K10 control.

2.10 *In vitro* evaluation of the candidacidal activity of a synthetic killer decapeptide (KP)

Recombinant single-chain fragment variable (scFvH6) anti-idiotypic antibodies functionally mimicking the activity of PaKT (Section 8.1) have been produced by idiotypic vaccination with a mouse monoclonal antibody (mAbKT4) that neutralised PaKT (Magliani *et al*, 1997a). The sequencing of scFvH6 was performed according to the conventional Sanger method (Sanger *et al*, 1977; Sanger *et al*, 1992). A series of decapeptides belonging to the scFvH6 sequence were synthesized with special regard to CDR regions. A synthetic decapeptide (P6, sequence: EKVTMTCSAS) pertaining to CDR-L1 and exerting a remarkable candidacidal activity *in vitro*, was selected for analysis by alanine scanning in order to identify the functional contribution of each residue. In Table 2.1, the *in vitro* candidacidal activities of P6 and peptides obtained by its alanine scanning are compared with those of a scramble peptide (SP), properly synthesized as an altered sequence of P6 (SP: MSTAVSKCET), which showed no *in vitro* candidacidal activity at all, and which was used as a control. All the substituted decapeptides retained some activity, but the one with alanine (A) replacing (E), named KP (Table 2.1, decapeptide number 2: AKVTMTCSAS), showed surprisingly greater dose-dependant activity. The decapeptide KP was then selected and synthesized (Neosystem, Strasburg, France) (Polonelli *et al*, 2003) to be used in this study.

2.10.1 Candidacidal activity of KP

The candidacidal activity of KP was evaluated *in vitro* by a cfu-determining assay on randomly selected strains of *Candida* spp. isolated and identified from each patient group (23 strains from DM patients from Parma, 24 from DM patients from London and 24 strains from controls) (Chapter 8, Table 8.1). *Candida* strains were re-grown in M199 medium at 37°C for 2 h.

Candida cells were counted using a haemocytometer and 10 µl of yeast cell suspension (2-3 × 10^2 cells) was added to 90 µl of sterile water containing synthetic KP to obtain a final yeast concentration of 20-30 cells/ml. The final concentrations of KP were 100 and 20µg/ml which were incubated for 6 h at 37°C. The same concentrations of the SP, properly synthesized as an altered sequence of the KP, were used as controls. After incubation with the respective reagents, the fungal cells were dispensed and streaked on the surface of SDA plates, which were then incubated at 30°C and the resulting cfu enumerated after 48 h. Each experiment was performed in triplicate.

In order to establish the minimal fungicidal concentration, corresponding to 100% killing of *C. albicans* cells, the above mentioned cfu assay was also performed in the presence of scalar concentrations of KP (15 µg/ml, 10 µg/ml, and 5 µg/ml) in comparison with SP on four of the *Candida* strains investigated in this study (two *C. albicans* subgroup A, one susceptible and one resistant to itraconazole; two non-*C. albicans*, one susceptible and one of intermediate resistance to azoles) (Chapter 8, Table 8.4). The strains selected were previously tested with KP at the above reported concentrations (100 and 20 µg/ml) with 100% killing activity. Each experiment was performed in triplicate.

The colonies of each *Candida* strain investigated in this study, which grew on SDA plates in each experiment, were cultured on SDA for 24 h, re-suspended in M199

medium and re-incubated with 90 µl of KP (final concentration 100 and 20 µg/ml), as described above, in order to establish whether the parental *Candida* cfu were clones intrinsically resistant to the KP or if they only exhibited transient resistance to KP under the experimental conditions adopted in this study.

Table 2.1: *In vitro* activity against *C. albicans* of the products obtained by alanine scanning of the decapeptide P6

(Magliani et al, 2004)

Decapeptide	100 μg/ml	25 μg/ml	6.25 μg/ml
1.EKVTMTCSAS	5.7±0.2*	29.8±10.6	67.1±13.8
2.AKVTMTCSAS	0*	0*	0*
3.EAVTMTCSAS	9.93.3	42.7±2.0	53.4±7.0
4.EKATMTCSAS	9.3±2.4*	19.7±2.9	60.1±5.2
5.EKVAMTCSAS	9.2±4.1*	26.6±4.2	63.2±4.8
6.EKVTATCSAS	0.1±0.1*	10.1±3.0*	40.4±16.0*
7.EKVTMACSAS	52.9±3.9	55.5±3.7	58.1±8.2
8.EKVTMTASAS	55.7±10.2	59.7±4.8	64.3±6.7
9.EKVTMTCAAS	2.6±0.5*	23.1±3.6*	72.9±7.4
10.EKVTMTCSAA	11.9±0.6*	32.9±0.8*	70.3±9.1
11.MSTAVSKCET	100	100	100

Decapeptide 1 is P6; decapeptides 2-10 are derived from P6 by alanine scanning; decapeptide 2 is KP (highlighted in yellow); 11 is the scramble decapeptide (SP) derived from P6 and used as a control. The activity of each decapeptide is expressed as a percentage of growth in comparison with the control in a cfu assay, essentially carried out as previously described (Section 2.10.1) (Polonelli *et al.* 2003), after incubation for 6 h at 37°C with the respective reagents

Statistically significant differences ($p<0.005$) in cfu counts in comparison with the control (each test performed in triplicate) are indicated by *

Statistical significance was assessed using the two-tailed Student's t test.

CHAPTER 3

Patient demographics

3.1 Introduction

A large epidemiological study (King *et al*, 1998) of the prevalence, numerical estimates and projections of the global distribution of DM between 1995 and 2025 has recently been published (King *et al*, 1998). Diabetes mellitus is a common multi-systemic disorder (Section 1.8) whose global prevalence in adults was estimated to be 4% in 1995 (135 million adults affected) and projected to rise to 5.4% by the year 2025 (300 million adults affected) (King *et al*, 1998). The prevalence is higher in developed than in developing countries and is expected to remain so until 2025. However, proportional increase will be greater in developing countries (48% from 3.3 to 4.9%), particularly in China and India, whilst in Europe, the increase will be the lower (26%).

In developed countries the majority of persons with DM are currently over 65 years of age and this is unlikely to change. Furthermore, on a global scale, there are more women with DM than men (73 vs 62 million as estimated in 1995). The excess of female patients is more pronounced in developed countries (31 vs 20 million) and will probably decrease slightly in European countries by the year 2025. Likely explanations for this female predominance are greater longevity of women and the existence of differential distribution of risk factors (especially diet, physical inactivity and central obesity) (King *et al*, 1998).

Other epidemiological studies have identified geographical variations between different populations (Diamond, 2003) in the incidence of the two types of DM. Type 2 DM, the more frequent form of disease, is reportedly (Zimmet *et al*, 2001)

becoming more common in the young and its incidence is spreading among many different populations (Asian Indians, Chinese, Japanese, Aboriginal Australians, Hispanic Americans and Afro-Americans) (Zimmet *et al*, 2001; Diamond, 2003). The epidemiology of type 1 DM in Europe has recently been studied by EURODIAB (EURODIAB ACE Study Group, 2000) in a large number of European Diabetic Centres in order to evaluate geographical variations and environmental factors affecting this form of DM. Preliminary data from this study confirmed large differences in type 1 DM incidence rates within different European countries. It is more likely that these differences are due to environmental factors such as perinatal infections or a rapid growth rate in early life than to genetic differences, as Europeans are more relatively genetically homogeneous (Cavalli-Sforza & Piazza, 1993; EURODIAB ACE Study Group, 2000).

Diabetic patients have been regarded by several authors over the years as a group at possible risk of developing oral candidal infections (Section 1.8.9.1.1). These studies have focussed on a number of variables that could promote a switch from a commensal state of *Candida* spp. carriage in the oral cavity to a pathogenic state in DM patients. In particular, systemic factors such as diabetic control (usually expressed as a fraction of glycosylated haemoglobin, HbA_{1c}), the duration of disease and the presence of long-term diabetic complications have been investigated (Aly *et al*, 1995; Dorocka-Bobkowska *et al*, 1996; Willis *et al*, 1999; Manfredi *et al*, 2002). In addition, local factors, such as the presence of dentures or tobacco habits, have been assessed for promoting adhesion to the mucosal epithelia. The relationship between the presence of DM and the development of oral candidal infections is still unclear (Section 1.8.9.1.1). One of the possible reasons for the discrepancies between these studies is the different sample populations investigated (often type 1 only or type 2 DM only) or the different measure of glycaemic control used (degree of glycosuria,

blood glucose levels, HbA_{1c}). Even different sampling techniques have been used to quantify oral *Candida* colonisation (Section 1.6) and have reported widespread colonisation (Williams & Lewis, 2000).

Therefore, in order to clarify if there are variables that could promote an increase in oral candidal carriage or the risk of developing oral candidal infections in DM patients, it is necessary to use the same methodologies to simultaneously evaluate a large number of comparable DM patients affected by the two types of DM and a group of non-DM subjects. An initial comparison between the two DM groups is required to evaluate the possible differences related to gender, age, type of DM, glycaemic control and geographical variations that may influence the carriage of oral yeasts (or *Candida* spp.) and the development of oral candidosis. Furthermore, the presence of dentures could characterise the dental management of a particular DM (and elderly) population, reflecting the social behaviour of that particular group of patients.

Local oral factors such as the presence of dentures or smoking habits may also directly affect the oral microflora and thus the likelihood of oral *Candida* infections.

Aim: The aim of this chapter was to ascertain whether there were differences between the studied patient cohorts in terms of gender, age, tobacco usage, denture presence and diabetic status. The relationship between any observed differences in these parameters and the occurrence of DM or the patient's geographical origin was ascertained.

Hypothesis: That there are differences in terms of gender or age, tobacco habits, type of DM or glycaemic control and presence of dentures (either partial or full) that may

attribute either to the presence of DM, or correlate with the patient's geographical location.

3.2 Methods

A medical history as well as a detailed oral examination for each enrolled patient was obtained (Section 2.1).

Analyses were made to assess the possible differences between DM and non-DM subjects, irrespective of locality, which may be attributed to patient gender, type of DM, age of patients, smoking habits, level of haemoglobin glycosylation and presence of dentures (either partial or full).

Fisher's exact test and Chi square test were used for statistical analysis of categorical data, and the Student T test or Anova were used to analyse numeric data according to the postulates of each test. Differences among or between groups were deemed significant when the probability (p) was less than or equal to 0.05.

3.3 Results

3.3.1 Diabetic patients affected by oral candidosis

During the course of this study, only 7/242 (2.8%) patients attending these two geographically different outpatient diabetic clinics had clinical signs of oral candidosis (5/242 were observed during London Diabetic Clinic appointments and 2/242 during Parma Diabetic Clinic appointments). Five of the affected patients were female and two were male. Although all were asymptomatic, 4 patients had clinical signs of angular cheilitis (three patients with type 2 DM and one with type 1 DM) associated with *Candida*, one patient with type 2 DM showed chronic erythematous candidosis of the palate and finally two type 2 DM patients had clinical signs of pseudomembranous candidosis (Table 3.1).

The six patients with type 2 DM with signs of oral candidosis were all over 60 years and were wearing an oral prosthesis (five had full dentures and one had a partial prosthesis). One of these type 2 DM patients was a tobacco user. The only patient affected by type 1 DM was younger than 60 years old (30 years old), dentate and a tobacco user.

The diagnosis of DM had been made more than 10 years previously to the enrolment in this study for 5 of the 7 DM patients affected by oral candidosis. Glycaemic control, assessed by HbA_{1c}, was used to sub-classify patients into three comparable groups according to the diabetology guidelines (Section 2.1.1). The HbA_{1c} levels were over 8.5% in 2/7 patients (one affected by type 1 DM and one by type 2 DM), between 7.5% and 8.5% in 3/7 patients (two affected by type 2 DM and one by type 1 DM) and under 7.5% in 2/7 patients.

Four out of the seven patients affected by oral candidosis had no long-term diabetic complications recorded (1/7 type 1 DM and 3/7 type 2 DM); the others patients (all affected by type 2 DM) had neuropathy and/or retinopathy, but none had signs of nephropathy.

The oral yeast load (Section 2.3) was greater than 100 cfu/ml in 6/7 (85.7%) patients and lower than 100 cfu/ml only in 1/7 (14.2%) patients. All these patients had one oral *Candida* species each: five isolates were further identified (Section 2.4.2, 2.4.2.1) as *C. albicans* genotype A and two as *C. parapsilosis* (Table 3.1).

Due to their low number (2.8%, 7/249), these patients were excluded from further statistical analysis in the present study.

3.3.2 Diabetic patients without oral candidosis

A total of 249 patients affected by DM (77/249 affected by type 1 DM and 172/249 by type 2 DM) were enrolled in this study. Two hundred and forty-two patients (125

male, 51.6%; 117 female, 45.8%; mean age 57.5 years, SD 15.6) were free of oral candidosis and were used for statistical analysis in this study (Tables 3.2-3.3).

Of the 242 DM patients without oral candidosis, DM type 1 had been previously diagnosed for 75 (30.9%) patients (56/75 from London and 19/75 from Parma) and the remaining 167/242 (69%) had been diagnosed with type 2 DM (81/167 from London and 86/167 from Parma).

Of the 167 DM patients with type 2 DM, 23 (13.7%) were being treated with a hypoglycaemic diet only, 108 (64.6%) were being treated with oral hypoglycaemic agents and 36 (21.5%) were using insulin. Only 10 out of these 36 (27.7%) were using a combined therapy of insulin and oral hypoglycaemic agents.

The diagnosis of DM was made more than 10 years before the present oral examination for 117/206 (56.7%) DM patients and less than 10 years for 89/206 (43.2%). For 36 DM patients (all enrolled in London), it was not possible to establish the date at which DM was first diagnosed.

Glycaemic control from each patient (HbA_{1c}) was used to sub-classify patients into three comparable groups according to the diabetology guidelines (Section 2.1.1). The HbA_{1c} levels were >8.5% in 105/242 (43.3%) patients (64/105 from London and 41/105 from Parma), between 7.5% and 8.5% for 62/242 (25.6%) patients (21/62 from London and 41/62 from Parma) and <7.5% for 75/242 (30.9%) patients (52/75 from London and 23/75 from Parma).

Retinopathy was the most common long-term diabetic complication recorded in 72/242 (29.7%) patients (41/72 from London, 31/72 from Parma), followed by neuropathy, recorded in 58/242 (23.9%) patients (37/58 from London, 21/58 from Parma) and nephropathy recorded in 15/242 (6.2%) of the DM patients (4/15 from London and 11/15 from Parma).

At the time of the oral examination, the majority of the DM patients enrolled (142/242; 58.6%) were dentate (or at least had some teeth in their oral cavity) and 100/242 (41.3%) were wearing partial or full dentures. Out of the 100 patients who were wearing a denture at the time of examination, 50/100 (50%) were wearing a full oral prosthesis (17/50 from London and 33/50 from Parma) and 50/100 (50%) a partial one (23/50 from London and 27/50 from Parma). Five patients (2%), out of the 242 who received oral examination, complained of a dry mouth although no signs of dryness or papillary atrophy were detected.

None of the DM patients enrolled in the present study were taking either topical or systemic antifungal drugs at the time of oral examination or had done so in the previous 6 months.

The majority of these DM patients were non-smokers (203/242, 83.8%). Of the 39 smokers, 36 patients were tobacco smokers, 2 were betel-nut chewers and one patient was a pipe smoker. These last three patients were all enrolled from the London Diabetic Clinic.

3.3.3 Non-DM control subjects from London, UK

A total of 130 non-DM subjects (63 males, 48.4%; 67 females, 51.5%; mean age 50.74 years, SD 17.67) (Section 2.1.3) were enrolled as a control group. It was not possible to establish the age of twelve of these patients. Of the remaining 118, 84 (71.1%) were less than 60 years old. None of these patients had oral candidosis, nor were they immunocompromised or taking any form of systemic or topical medication.

The majority of the control subjects were dentate 87/130 (66.9%) and 43 out of 130 (33%) were wearing dentures (either full or partial). Twenty-one of the 43 (48.8%) had a full denture and 22/43 (51.1%) were wearing a partial oral prosthesis.

One hundred and three of the 130 (79.2%) control patients were non-tobacco smokers and only 27/130 (20.7%) tobacco users. Only one of the tobacco users was a betel-nut chewer, the other twenty-six were tobacco smokers.

3.4 Results of the comparison between the three groups of patients

The comparison between the DM patients group and the non-DM subjects showed that there were no differences in the gender distribution and tobacco habits among these populations (Table 3.2). Although for 12 of the non-DM subjects it was not possible to establish their date of birth (special needs patients), significant differences in age were observed between the two groups (Table 3.2). In particular, DM patients were older than non-DM control subjects ($p=0.0004$).

However, a number of significant differences were observed between the two groups of DM patients (Table 3.3). Diabetic patients from Parma were older ($p=0.001$) than those from London, and more patients from London than Parma had type 1 DM ($p=0.001$).

Furthermore, although there was no significant difference in the presence of diabetic complications and duration of disease in DM patients (from London and Parma) ($p=0.72$), London patients had a higher incidence of neuropathy and a lower incidence of nephropathy ($p<0.0001$) compared with those from Parma. More specifically, there were fewer DM patients from London who developed nephropathy after 10 years of DM disease in comparison to DM patients from Parma ($p=0.02$); higher levels of $HbA1_c$ (>8.5%) were observed in the patients from London ($p<0.001$) and more patients from Parma were denture wearers ($p<0.0001$) (Table 3.3).

3.5 Discussion

The aim of this study was to establish whether there were differences in terms of gender, age, tobacco habits, presence of dentures and diabetic status between the patients cohorts assessed in the present study. Although only small differences were observed between DM patients when compared with non-DM subjects, the two groups of DM patients evaluated did differ in a number of aspects relating to DM.

Within the confines of this study, it has not been possible to establish the exact reason for these differences. They may simply reflect differences between clinical practices in the two regions or perhaps an unknown geographic variation in the frequency and/or clinical manifestation of DM.

Diabetic patients from Parma were older ($p=0.001$) than those from London (Table 3.3). This age variation may reflect the difference in the type of DM that each group had. Type 1 DM, which often develops before 30 years of age (Section 1.8), was observed mostly in the DM patients from London ($p<0.001$).

The principal trend observed in the present study was that patients from London were more likely to have the type 1 disease, use insulin, and experience neuropathic complications of DM than those from Italy.

Wide geographical variations have been described in the incidence of type 1 DM particularly within Europe, with extremely high incidences observed in Finland, Sweden, Denmark, Norway and the United Kingdom and much lower incidences in France and Italy, with the exception of Sardinia (Bell & Hockaday, 1996; Diamond, 2003). The analysis made on the two geographically different DM populations may confirm the gradient of disease from North to South of Europe, although of course it would be more relevant to evaluate large DM populations from the two countries to assess the exact different incidences of the two types of DM.

It has been hypothesized that patients with DM are prone to fungal infections (Bai *et al*, 1995; Dorocka-Bobkowska *et al*, 1996; Willis *et al*, 1999; Willis *et al*, 2001). Although the patients with DM from the UK investigated in the present study were different in many aspects of their disease from the DM patients from Italy, both groups demonstrated a low incidence of oral *Candida* infections. During the course of this study only 7/249 (2.8%) DM patients that had a detailed oral examination had clinical signs of oral candidosis. This low percentage of oral candidal infections in DM patients seems to contrast with data reported elsewhere (Dorocka-Bobkowska *et al*, 1996; Abu-Elteen & Abu-Alteen, 1998; Willis *et al*, 1999; Guggenheimer *et al*, 2000b; Willis *et al*, 2000b), and cannot simply be attributed to clinician awareness (all oral examinations in the present study were carried out by a qualified, experienced dentist) or diabetic control. Locality may influence the incidence of this disease, although the reasons for this are difficult to pinpoint (Manfredi *et al*, 2002).

The development of oral infections by *Candida* spp. is a complex pathogenetic process that involves both the host and the yeast. In the small group of DM patients affected by oral candidosis, different factors may have promoted the infection. Local factors, such as the presence of a dental prostheses (6/7, 85.7% of the patients were wearing a denture at the time of the oral examination), systemic factors, such as poor diabetic control (5/7, 71.4% of the patients had a HbA_{1c} greater than 7.5%) or impaired host immune defences, not investigated in this study, could have all promoted *Candida* spp. pathogenicity in the oral cavity.

These patients may also have had partial disruptions of oral mucosal barriers that had provided sites for local infections or a disruption of the ecological balance of the host's oral microflora. Significantly, the diabetic status or general conditions of these 7 DM patients did not obviously differ from the other 242 DM patients that showed no signs of oral candidosis.

From the analysis conducted on these groups of patients, it was possible to establish that there were no significant differences in the demographics of DM patients and non-DM subjects evaluated as a control group, but there were interesting variations in the diabetic disease of the two geographically different DM populations.

Table 3.1: Diabetic patients affected by oral candidosis

Variables investigated	London DM patients	Parma DM patients	Total DM patients
Gender			
Male	4/5	0/2	4/7 (57.1%)
Female	1/5	2/2	3/7 (42. 9%)
Age			
>60 years	4/5	2/2	6/7 (85.7%)
<60 years	1/5	0/2	1/7 (14.3%)
Denture status			
Dentate	4/5	0/2	4/7 (57.1%)
Dentures	1/5	2/2	3/7 (42.9%)
Tobacco usage			
Tobacco users	1/5	1/2	2/7 (28.6%)
Non-tobacco users	4/5	1/2	5/7 (71.4%)
DM type			
Type 1 DM	1/5	0/2	1/7 (14.3%)
Type 2 DM	4/5	2/2	6/7 (85.7%)
Time since diagnosis of DM			
DM < 10 years	0/5	2/2	2/7 (28.6%)
DM > 10 years	5/5	0/2	5/7 (71.4%)
Glycaemic control			
$HbA1_c$ <7.5%	2/5	0/2	2/7 (28.6%)
7.5 %< $HbA1_c$ <8.5%	1/5	2/2	3/7 (42.8%)
$HbA1_c$ >8.5%	2/5	0/2	2/7 (28.6%)
DM complications			
Neuropathy	3/5	0/2	3/7 (42.9%)
Retinopathy	2/5	0/2	2/7 (28.6%)
Nephropathy	0/5	0/2	0/7 (0%)
Oral *Candida* carriage			
<100 cfu/ml	1/5	2/2	3/7 (42.9%)
>100 cfu/ml	4/5	0/2	4/7 (57.1%)
***Candida* genotype**			
C. albicans A	4/5	1/2	5/7 (71.4%)
C. albicans B	0/5	0/2	0/7 (0%)
C. albicans C	0/5	0/2	0/7 (0%)
C. parapsilosis	1/5	1/2	2/7 (28.6%)

Table 3.2: Demographics of the patients with and without DM

Demographics	Total DM patients	Non-DM subjects	p value
Male	125/242 (51.7%)	63/130 (48.5%)	
Female	117/242 (48.3%)	67/130 (51.5%)	0.587
Age (mean, SD^)	57.5 (15.6)	50.8 (17.5)	**0.0004**
<60 years old	111/242 (45.9%)	84/118* (71.2%)	
>60 years old	131/242 (54.1%)	34/118* (28.8%)	**<0.0001**
Dentate	142/242 (58.7%)	87/130 (66.9%)	
Dentures	100/242 (41.3%)	43/130 (33.1%)	0.146
Tobacco users	39/242 (16.1%)	27/130 (20.8%)	
Non-tobacco users	203/242 (83.9%)	103/130 (79.2%)	0.319

Statistical analysis was performed using Fisher's and Chi-square parametric tests
SD^ = standard deviation
*Twelve of the non-DM subjects did not give their date of birth (special needs patients)

Table 3.3: Comparison of patient gender, age, type and control of DM and denture status between patients with DM from two different geographic locales

Variables investigated	London DM patients	Parma DM patients	p value
Gender			
Male	70/137 (51.1%)	55/105 (52.4%)	
Female	67/137 (48.9%)	50/105 (47.6%)	0.89
Age, mean (SD^)	54.8 (16.2)	61.1 (14.1)	**0.001**
DM type:			
Type 1	56/137 (40.9%)	19/105 (18.1%)	
Type 2	81/137 (59.1%)	86/105 (81.9%)	**0.0001**
Time since diagnosis of DM			
<10 years	35/101** (34.7%)	54/105 (51.4%)	
>10 years	66/101** (65.3%)	51/105 (48.6%)	0.08
Complications of DM			
Neuropathy	37/137 (27%)	11/105 (10.5%)	
Retinopathy	41/137 (29.9%)	31/105 (29.5%)	
Nephropathy	4/137 (2.9%)	21/105 (20%)	**<0.0001**
Diabetic control (HbA_{1c})			
HbA_{1c} <7.5%	52/137 (38%)	23/105 (22%)	
HbA_{1c} >7.5 - <8.5%	21/137 (15.3%)	41/105 (39%)	
HbA_{1c} >8.5%	64/137 (46.7%)	41/105 (39%)	**<0.0001**
Denture status			
Dentate	97/137 (70.8%)	45/105 (42.9%)	
Dentures	40/137 (29.2%)	60/105 (57.1%)	**<0.0001**

Statistical analysis was performed using Fisher's exact and Chi square parametric tests
SD^ = standard deviation
** For 36 of the London DM patients it was not possible to establish the date when their DM was diagnosed

CHAPTER 4

Yeast isolation and species identification

4.1 Introduction

Candida spp. have frequently been isolated from the oral cavity of patients affected by DM (Darwazeh *et al*, 1990). Recently, it has been reported that up to 77% of insulin-treated DM patients harbour *Candida* spp. in their oral cavities (Willis *et al*, 2000b). A number of factors have been associated with the oral carriage of yeast in DM patients, such as the type and duration of disease and the degree of glycaemic control (Hill *et al*, 1989; Darwazeh *et al*, 1990). Oral factors, such as denture wearing, have been postulated to contribute to yeast colonization in DM patients (Dorocka-Bobkowska *et al*, 1996). Some controversy therefore remains regarding the role of DM in yeast colonisation in the oral cavity.

Candida dubliniensis, a newly described species of *Candida* mainly isolated from immunocompromised patients (Sullivan & Coleman, 1997; McCullough *et al*, 1999a), has recently been isolated from a group of insulin-treated patients with DM in Ireland (Willis *et al*, 2000b). Utilizing simple molecular methodologies, it is now possible to delineate *C. dubliniensis* from *C. albicans*. Furthermore, it is possible to separate *C. albicans* genotypically into subgroups which have been shown to vary geographically and which may be implicated in the development of antifungal resistance (McCullough *et al*, 1999b; McCullough *et al*, 1999a; Manfredi *et al*, 2002).

Hence, there is a need to extend our understanding of oral yeasts in DM patients, particularly with respect to species of yeasts present and clinical parameters

influencing oral yeast colonisation. Furthermore, the advances in relevant molecular biological techniques now allow a more detailed analysis of the relationship between DM and oral *Candida* carriage.

Aim: The aim of this study was to isolate, identify and genotype the *Candida* isolates from two groups of patients affected by DM from London, UK and Parma, Italy, and from a healthy non-DM population.

Hypothesis: That there are differences in the frequency of isolation, degree of colonisation, species or genotypic distribution of oral yeast that can be attributed to either presence of DM or patient geographic location.

4.2 Methods

Oral yeasts isolated from DM patients from London (Section 2.1.1), Parma (Section 2.1.2) and from control non-DM London control subjects (Section 2.1.3) were analysed in this part of the study. Oral yeasts were isolated using the oral mouth rinse technique (Section 2.2), enumerated (Section 2.3), defined at species level (Section 2.4) and genotyped (Section 2.5) as outlined previously.

Fisher's exact test and Chi square test were used for the statistical analysis of categorical data, numeric data was analysed by the Student T test or ANOVA according to the postulates of each test, and differences within or between groups were considered significant when the probability (p) was less than or equal to 0.05.

4.3 Results

4.3.1 Oral yeast isolation and recovery from all patients

The present study assessed the oral candidal load of DM and non-DM subjects. As reported (Section 3.3.2), the majority of the patients (242/249, 97%) had no clinical evidence of oral candidosis. Saliva was collected from the patients using the concentrated oral rinse technique (Section 2.2) and the yeast load was expressed as cfu per ml of mouth rinse (Section 2.3). This quantitative evaluation showed that whilst there was a general mean of approximately 100 cfu/ml, actual loads varied extensively between patients. An oral carriage rate of 100 cfu/ml was however selected an "internal cut-off" and used as an indicator of possible local or systemic factors that could have influenced the oral *Candida* carriage in these populations.

A total of 372 patients were enrolled in this study. One hundred and thirty of these were non-DM control subjects (Section 2.1.3) and the remaining 242 DM patients were individuals attending the clinics in London and Parma (Section 2.1.1-2.1.2). Of the 372 patients, 225 (60.4%) were found to carry *Candida* their oral cavity and the oral yeast load was <100 cfu/ml in 117/225 (52%) of these patients. One hundred and fifty-two of the 242 DM patients (62.8%) had *Candida* in their oral cavity. For 93 (61.1%) of these patients, the yeast load was <100 cfu/ml (Table 4.1). Two of the 152 DM patients (both from Parma) who had *Candida* in their oral cavity, were found to have 2 *Candida* strains resulting in a total of 154 *Candida* isolates.

Of the 130 non-DM subjects, 73 (56.2%) had oral yeast. Of note, out of these 73 patients two had 2 *Candida* strains isolated, resulting in a total of 75 isolates.

The yeast load was <100 cfu/ml in 24 (32.9%) of these non-DM subjects (Table 4.1).

4.3.1.1 Phenotypic and genotypic identification of *Candida* isolates

4.3.1.1.1 Diabetic patients

Phenotypic methods identified 127/154 isolates (82.5%) as either *C. albicans* or *C. dubliniensis*. Genotypic methods further distinguished these 127 isolates into 99 *C. albicans* genotype A, 17 *C. albicans* genotype B, 5 *C. albicans* genotype C and 6 *C. dubliniensis*. The 27 remaining isolates were identified by genotypic methods as non-*C. albicans/dubliniensis* species and these comprised of 12 *C. glabrata*, 2 *C. lusitaniae*, 3 *C. tropicalis*, 8 *C. parapsilosis*, 1 *C. krusei* and 1 *C. guilliermondii*. *Candida dubliniensis* was isolated from the oral cavity of 6 out of 152 (3.9%) DM patients who harboured oral yeasts. Four of these six patients were female and all had some natural teeth (two patients were partially dentate whilst the other three were fully dentate) (Table 4.1).

As reported in Section 4.3.1, two Italian DM patients had two *Candida* stains each (154 isolates in total). One of these patients had two different *C. albicans* genotypes (A and B), whilst the other patient had one *C. albicans* genotype B and one *C. glabrata*.

4.3.1.1.2 Non-DM subjects

Sixty-five out of 75 isolates from the oral cavities of the non-DM subjects were identified as either *C. albicans* or *C. dubliniensis* using phenotypic and genotypic methods. Genotyping identified 46 *C. albicans* genotype A, 8 *C. albicans* genotype B, 2 *C. albicans* genotype C and 9 *C. dubliniensis* (Table 4.1). The 10 remaining isolates were genotypically identified as non-*C. albicans* spp.: 5 *C. glabrata*, 1 *C. lusitaniae*, 1 *C. guilliermondii* and 3 *C. krusei* (Table 4.1).

Two non-DM subjects harboured two different isolates in their oral cavity. One of these patients had *C. glabrata* and *C. krusei* whilst the other patient had *C. lusitaniae* and *C. krusei* isolated simultaneously. *Candida dubliniensis* was isolated from the oral cavities of nine of the 75 (12%) control patients who harboured oral yeast. Six of

these patients were male and all had some natural teeth (eight patients were fully dentate and one was partially dentate) at the time of the oral examination. None of these patients had mixed yeast species in their oral cavity.

4.3.1.2 Factors influencing oral *Candida* carriage

4.3.1.2.1 Diabetic patients and non-DM subjects

No significant difference was evident in the isolation of *Candida* from the oral cavity of DM patients with either type 1 or type 2 DM ($p=0.15$) (Table 4.3). Furthermore, the frequency of isolation and oral candidal load (cfu/ml) within the DM patients was not influenced by patient age, gender, duration or glycosylated haemoglobin levels ($p>0.05$) (Tables 4.2-4.3). Statistically significant ($p=0.001$) (Table 4.3) was the finding that DM patients affected by nephropathy had fewer oral yeasts than DM patients affected by other long term diabetes complications. However, no difference in the occurrence of diabetic complications and oral candidal load was observed ($p>0.05$). Furthermore, significantly ($p=0.0001$) more denture-wearing DM patients had oral *Candida* (Table 4.2) compared with dentate individuals. There was however no significant difference observed in the actual candidal load between these patient groups. No association ($p>0.05$) was evident with either the frequency or load of yeast carriage and patient smoking habits or DM status (Tables 4.2-4.3).

Neither the frequency nor load of *Candida* carriage was influenced by age, gender, smoking habits or presence or absence of dentures ($p>0.05$) in non-DM subjects.

4.3.1.2.2 All patients

No significant difference was found in the isolation of *Candida* from the oral cavity of all patients investigated and patient gender, age or tobacco habits ($p>0.05$) (Table 4.2). Statistically significant ($p<0.0001$) was the finding that a higher number of

denture wearers (105/143; 73.4%) had oral *Candida* compared with non-dentate patients (120/229, 52.4%) (Table 4.2).

There was no significant association ($p>0.05$) between oral candidal load and patient gender, age, presence or absence of dentures and tobacco smoking status (Table 4.2).

4.3.1.3 Factors influencing *Candida* spp. and *C. albicans* genotype recovery

4.3.1.3.1 Diabetic patients and non-DM subjects

The species of oral yeast present and genotypic distribution of *C. albicans* were not influenced by patient age, gender, duration or type of DM, glycosylated haemoglobin levels or diabetic complications ($p>0.05$) (Tables 4.4-4.5).

DM patients with dentures, (either partial or full) had more non-*C. albicans* (23/78, 29.5%) isolated from their mouths than dentate patients (10/76, 13.1%) ($p=0.01$) (Table 4.4).

There was no significant correlation between the type of *Candida* isolated and non-DM subjects' age, gender, smoking habits, denture wearing status ($p>0.05$) (Table 4.2).

4.3.1.3.2 All patients

The species of oral yeast present were not influenced by patient gender ($p>0.05$). However, the frequency of non-*C. albicans* spp. isolation was significantly higher in elderly patients (>60 years old) (30/107, 28%) compared with younger patients (17/109, 15.6%; $p=0.03$) (Table 4.4).

No significant association was found between the type of yeast species isolated and tobacco habits in any of the patients investigated ($p>0.05$). Furthermore, no significant association was observed in the genotypic distribution of *C. albicans* and patient gender or age, presence or absence of dentures or tobacco habits ($p>0.05$).

4.3.2 Comparison between DM patients and non-DM subjects

4.3.2.1 Oral yeast growth and degree of colonisation (cfu/ml)

A high yeast load (>100 cfu/ml) was significantly (p=0.001) more frequent in non-DM subjects (49/73, 67.1%) compared with DM patients (59/152, 38.8%) (Table 4.1). The oral carriage rate was however significantly greater in elderly DM patients (>60 years) (88/152, 57.8%; p=0.0002) compared with their non-DM equivalents (10/64, 15.6%). Interestingly, comparison of oral yeast loads in younger patients (<60 years) revealed that significantly more (p=0.03) of the non-DM subjects (28/45, 62.2%) had higher *Candida* loads (>100 cfu/ml) compared with their DM equivalents (19/64, 29.6%).

Comparing all DM patients and non-DM subjects, revealed that there was no statistical differences in oral yeast carriage rate and there was similarly no statistically significant relationship between yeast load and patient gender, tobacco habits or denture wearing status (p>0.05).

4.3.2.2 Species of oral yeasts isolated and genotype of *C. albicans*

There was a significantly higher number (p=0.04) of non-tobacco-using, DM patients (28/124, 22.5%) with non-*C. albicans* spp. isolated compared with their non tobacco-using counterparts (11/57, 19.3%).

No differences were observed in the isolation of *C. albicans* genotypes between DM patients and non-DM subjects; however, more *C. parapsilosis* strains (8/33, 24%) were recovered from DM patients (p=0.02). In addition, a higher incidence (9/19, 47%) of *C. dubliniensis* isolates (p=0.054) was evident in the oral cavity of non-DM individuals (Table 4.1).

4.3.3. Comparison between the two geographically different DM populations

4.3.3.1 Oral yeast growth and degree of colonisation (cfu/ml)

There was no significant difference in the frequency of oral yeast carriage between the two geographical DM patient groups ($p>0.05$) (Table 4.5). However, a significantly higher number ($p=0.01$) of DM patients from London (40/83, 48.2%) had oral candidal loads >100 cfu/ml compared with Italian DM patients (19/69, 27.5%) (Table 4.5). Furthermore, oral *Candida* carriage and yeast load (cfu/ml) were not influenced by patient gender or age, presence or absence of dentures or tobacco habits ($p>0.05$).

All DM patients with complications attributable to poor long-term control of their disease had a higher incidence of yeast carriage in their oral cavity, (Parma retinopathy: 23/31; 74%; Parma nephropathy: 16/21; 76%; London neuropathy: 24/37, 64.8%; London retinopathy: 23/41; 56%; London nephropathy: 3/4; 75%) compared with those DM patients without complications irrespective of their geographic location. The exception to this was for patients from Parma who also suffered from neuropathy, and in this group oral yeasts were only isolated from a small number (7/39; 17.9%).

Interestingly, although patients with diabetic complications had higher oral candidal carriage rates, these patients (neuropathy: 15/31, 48.3%; retinopathy: 16/46; 34.7%; nephropathy: 8/19; 42.1%) did not have significantly higher candidal loads (cfu/ml) ($p=0.25$). No other significant differences were observed in oral *Candida* carriage or yeast load and diabetic status between the two groups of DM patients ($p>0.05$).

4.3.3.2 Species of oral yeasts isolated and genotype of *C. albicans*

Overall, there was no significant difference in the species of oral yeasts isolated or in the genotype of *C. albicans* among the DM patient populations ($p>0.05$). Similarly,

species of *Candida* or genotypic distribution of *C. albicans* were not influenced by patient age or gender ($p>0.05$) when the two populations of DM patients were compared.

Comparison of *C. albicans* genotypes and presence or absence of dentures showed that more *C. albicans* genotype A (44/49; 89.7%) were isolated from the oral cavities of London DM patients who were dentate than Parma dentate DM patients (10/17; 58.8%) ($p=0.0087$).

Interestingly, none of the tobacco-smoking DM patients from London had *C. albicans* genotype B or C isolated from the oral cavity ($p=0.015$). No other statistically significant differences were observed in the genotype of *C. albicans* and smoking habits among the DM populations ($p>0.05$)

Finally, no significant differences were observed in the species of oral yeasts isolated and type of DM, duration of disease, haemoglobin glycosylation level and diabetes complications among the two DM populations.

However, significantly more ($p=0.002$) *C. albicans* genotype A were isolated from the oral cavity of London DM patients affected by type 1 DM (25/28, 89.2%) than Parma DM patients with type 1 DM (3/8, 37.5%) (Table 4.6).

4.4 Discussion

It has been hypothesised that DM patients are prone to developing fungal infections (Dorocka-Bobkowska *et al*, 1996; Willis *et al*, 1999). The present study assessed, in detail, the oral colonisation, species and genotype of yeast in DM patients for variations that may be influenced by the type, disease progression, degree of control of DM and geographic location. The results of this investigation for two different groups of DM patients suggest that all these parameters have little or no influence on the oral yeast flora of DM patients (Manfredi *et al*, 2002).

In this study, *Candida* spp. were firstly detected on SDA plates (Section 2.2) and subsequently identified both by phenotypic (Section 2.4.1) and genotypic (Sections 2.4.2-2.4.2.1-2.5) techniques. Although it is well known that SDA rarely permits differentiation between *Candida* species within the same sample and other culture media are now available for a better discrimination of *Candida* spp. (Chapter 1, Section 1.6.1-1.6.2), the use of SDA was preferred in this study for the lower cost of the material compared with chromogenic media (*e.g.* CHROMagar). In addition, the differentiation between *Candida* spp. provided by CHROMagar (such as discriminating between *C. albicans* and *C. dubliniensis*) appears to decline with subculture or when isolates are stored. Moreover, the aim of this study was not to use the culture medium as an identification method but as a means of rapidly and easily assessing the presence of *Candida* isolates in the oral cavity of the patients studied. More sophisticated analyses were (Sections 2.4.2-2.4.2.1-2.5) used to identify the yeasts grown on the SDA plates.

The overall proportion of DM patients who had *Candida* isolated from their oral cavity in the present study (152/242; 62.8%) was similar to that previously reported (Fisher *et al*, 1987; Aly *et al*, 1992). However, contrary to previously published reports where oral yeast colonisation was higher in patients with type 1 DM (Aly *et al*, 1992; Bai *et al*, 1995), there was no significant difference in the present study between the number of type 1 and 2 DM patients whose oral cavity was colonised with yeast.

Similarly, it has previously been reported that the duration of DM and the degree of glycaemic control may influence the frequency and amount of oral *Candida* carriage (Rayfield *et al*, 1982; Hill *et al*, 1989; Willis *et al*, 1999; Guggenheimer *et al*, 2000b). However, the present results indicate that oral yeast carriage is not significantly

influenced by glycaemic control, duration or complications of DM. Nevertheless, the results of the present study indicated that the presence of dentures could affect both the amount and species of oral yeast present; therefore local oral factors, such as the presence of dentures would appear to be more important in terms of candidal carriage rates than any systemic aspects relating to DM.

Inconsistencies in reporting oral yeast prevalence in DM have, at least in part, been attributed to the variability of sampling methods (Tapper-Jones *et al*, 1981; Hill *et al*, 1989; Aly *et al*, 1992; Willis *et al*, 2000b). Comparisons between this study on two different groups of DM patients and one study on a healthy non-DM population allowed us to compare oral yeast colonisation using identical sampling, storage and laboratory methodologies. From this comparison, it would appear that DM patients are no more likely to harbour yeast in their oral cavity than healthy controls.

Interestingly, the trend observed in the present study that DM patients have a reduced candidal load compared to controls, would indicate that the presence of glucose in the saliva of DM patients (Darwazeh *et al*, 1991a; Dodds & Dodds, 1997) may have an inhibitory effect on yeast colonisation. Further studies are required to assess the level of salivary glucose present in these patients when simultaneously analysing yeast colonisation.

Combining the results for DM and control patients revealed that 48% of patients (108/225) without clinical oral candidosis had over 100 cfu/ml of yeast in their oral rinses. Interestingly, as reported by other authors (White *et al*, 2004), *Candida* levels as high as 3000 cfu/ml were evident in some patients with no signs of oral candidosis, while the patients affected by oral candidosis occasionally showed lower candidal cfu/ml than those observed of non-infected patients.

Correlation between the quantification of *Candida* in saliva and clinical status has long been debated in literature. Several studies (Samaranayake *et al*, 1986; MacFarlane, 1990) have proposed that the presence of elevated candidal numbers (2-3 x 10^3 cfu/ml) in saliva indicates infection rather than normal commensal carriage (600-800 cfu/ml) (McKendrick *et al*, 1967). However, other studies (Borromeo *et al*, 1992; Al Karaawi *et al*, 2002; White *et al*, 2004) have shown that simple quantification of *Candida* in saliva does not give any indication of health or disease. Although the determination of a cut-off limit for cfu between health and disease may serve as a useful clinical indicator, the results from this study, and from literature, show that quantitative oral yeast levels are not useful in determining the clinical status of the patient.

As reported in Chapter 3 (Section 3.3.1), during the course of this study, only 7/242 DM patients (2.8%) had clinical evidence of oral candidosis. This low incidence rate contradicts previous reports (Dorocka-Bobkowska *et al*, 1996; Abu-Elteen & Abu-Alteen, 1998; Guggenheimer *et al*, 2000b; Willis *et al*, 2000b) and cannot be explained simply by clinical awareness or diabetic control.

Candida dubliniensis, first isolated from HIV infected patients (Sullivan *et al*, 1995), has recently been recovered from the oral cavity of 58 out of 318 (18.2%) *Candida* positive, insulin-treated DM patients (Willis *et al*, 2000b). The prevalence reported in the present study (6/154 DM patients; 3.9%) is much lower. This may be due, at least in part, to the different culture methods used, as the present study assessed only the most prevalent species of yeast present in the oral cavity. A high percentage (14.1%, 45/318) of the previously described isolation of *C. dubliniensis* occurred when this yeast was found co-colonising the oral cavity with *C. albicans* or with other *Candida* spp. Whether this co-colonisation has any influence on clinical outcome has yet to be determined. If co-colonisation of *C. dubliniensis* with *C. albicans* in the previous

study (Willis *et al*, 2000b) is excluded, then only 4.1% of patients would be colonised solely with *C. dubliniensis*, which is similar to the findings in the present study (3.9%). Furthermore, 12% (9/75) of isolates from control patients in the present study were identified as *C. dubliniensis*, statistically greater (p=0.04) than DM patients (6/154) in the present investigation. Interestingly, all DM patients and the control population investigated in the present study that had *C. dubliniensis* isolated from their oral cavity were dentate (either fully or partially).

Global geographic variability in the genotypes of oral isolates of *C. albicans* has been observed (Clemons *et al*, 1997; McCullough *et al*, 1999b). The present study has not, however, found any association between a particular genotype of *C. albicans* and the DM type, disease progression and degree of DM control. It would be interesting to assess a larger portion of the genomes of these isolates to confirm this lack of genetic variability and to assess oral yeast isolates from a second group (geographically distant from London, UK) of control subjects. Nevertheless, the results of the present study suggests that there is nothing unique in the oral yeast flora of DM patients (Manfredi *et al*, 2002).

Table 4.1: Oral *Candida* growth and species isolated from patients with and without DM

Candida isolates	DM patients	Non-DM subjects	p value	Patients total
Growth	152/242 (62.8%)	73/130 (56.2%)		225/372 (60.5%)
No growth	90/242 (37.2%)	57/130 (43.8%)	0.22	147/372 (39.5%)
<100 cfu/ml	93/152 (61.2%)	24/73 (32.9%)		117/225 (52%)
>100 cfu/ml	59/152 (38.8%)	49/73 (67.1%)	**0.001**	108/225 (48%)
***C. albicans* total**	121/154*(78.6%)	56/75**(74.7%)		177/229 (77.3%)
Other species total	33/154*(21.4%)	19/75**(25.3%)	0.50	52/229 (22.7%)
***C. albicans* A**	99/121 (81.8%)	46/56 (82.1%)		145/177 (81.9%)
***C. albicans* B**	17/121 (14%)	8/56 (14.3%)		25/177 (14.2%)
***C. albicans* C**	5/121 (4.2%)	2/56 (3.6%)	0.98	7/177 (3.9%)
C. glabrata	12/33 (36.4%)	5/19 (26.3%)	0.54	17/52 (32.7%)
C. lusitaniae	2/33 (6.1%)	1/19 (5.3%)	1.00	3/52 (5.8%)
C. tropicalis	3/33 (9.1%)	0/19 (0%)	0.29	3/52 (5.8%)
C. parapsilosis	8/33 (24.2%)	0/19 (0%)	**0.02**	8/52 (15.4%)
C. krusei	1/33 (3%)	3/19 (15.8%)	0.13	4/52 (7.7%)
C. guilliermondii	1/33 (3%)	1/19 (5.3%)	1.00	2/52 (3.8%)
C. dubliniensis	6/33 (18.2%)	9/19 (47.3%)	0.054	15/52 (28.8%)

Statistical analysis was performed using Fisher's exact and Chi-Square parametric tests

* One hundred and fifty-two DM patients from Parma yielded a single *Candida* strain; 2 additional patients each yielded two strains (154 isolates in total)

** Seventy-one non-DM control patients yielded a single *Candida* strain; 2 additional control patients each yielded 2 strains (75 isolates in total)

Table 4.2: Oral *Candida* isolation and load (cfu/ml) in the different groups of patients evaluated

Patients	Growth	No growth	p value	<100 cfu/ml	>100 cfu/ml	p value
D/Male	78/125	47/125		48/78	30/78	
D/Female	74/117	43/117	0.89	45/74	29/74	1.00
D/<60 years old	64/113	49/113		45/64	19/64	
D/>60 years old	88/129	41/129	0.08	48/88	40/88	0.06
D/dentate	75/142	67/142		49/75	26/75	
D/dentures	77/100	23/100	**0.0001**	44/77	33/77	0.32
D/tobacco users	28/39	11/39		15/28	13/28	
D/non-tobacco users	124/203	79/203	0.27	78/124	46/124	0.39
C/ Male	36/63	27/63		10/36	26/36	
C/ Female	37/67	30/67	0.86	14/37	23/37	0.45
C/ <60 years old**	45/84	39/84		17/45	28/45	
C/ >60 years old**	18/34	16/34	1.00	6/18	12/18	0.77
C/ dentate	45/87	42/87		17/45	28/45	
C/ dentures	28/43	15/43	0.18	7/28	21/28	0.31
C/ tobacco users	17/27	10/27		6/17	11/17	
C/ non-tobacco users	56/103	47/103	0.51	18/56	38/56	1.00
Male total	114/188	74/188		58/114	56/114	
Female total	111/184	73/184	1.00	59/111	52/111	0.79
<60 years old total	109/197	88/197		62/109	47/109	
>60 years old total	106/163	57/163	0.06	54/106	52/106	0.41
Dentate total	120/229	109/229		66/120	54/120	
Dentures total	105/143	38/143	**<0.0001**	51/105	54/105	0.35
Tobacco users total	45/66	21/66		21/45	24/45	
Non-tobacco users total	180/306	126/306	0.16	96/180	84/180	0.50

Data was subdivided according to patient age, gender, presence or absence of denture and smoking habits
Statistical analysis was performed using Fisher's exact and Chi square parametric tests
D: DM patients; C: Non-DM control patients
** Of the twelve patients who did not give their date of birth (special needs patients), 10 harboured oral yeasts

Table 4.3: Oral *Candida* isolation and load (cfu/ml) in patients with DM

DM patients	Growth	No growth	p value	<100 cfu/ml	>100 cfu/ml	p value
D/Type 1 DM	42/75	33/75		27/42	15/42	
D/Type 2 DM	110/167	57/167	0.15	66/110	44/110	0.71
D/DM <10 years*	55/89	34/89		36/55	19/55	
D/DM >10 years*	75/117	42/117	0.77	46/75	29/75	0.71
D/neuropathy	31/76	45/76		16/31	15/31	
D/retinopathy	46/72	26/72		30/46	16/46	
D/nephropathy	19/25	6/25	**0.001**	11/19	8/19	0.48
D/HbA_{1c} <7.5%	46/75	29/75		30/46	16/46	
D/HbA_{1c} >7.5%; <8.5%	40/62	22/62		27/40	13/40	
D/HbA_{1c} >8.5%	66/105	39/105	0.92	36/66	30/66	0.34
D/L Type 1 DM	33/56	23/56		20/33	13/33	
D/L Type 2 DM	50/81	31/81	0.85	23/50	27/50	0.16
D/L DM <10 years*	22/35	13/35		10/22	12/22	
D/L DM >10 years*	39/66	27/66	1.00	22/39	17/39	0.43
D/L neuropathy	24/37	13/37		12/24	12/24	
D/L retinopathy	23/41	18/41		13/23	10/23	
D/L nephropathy	3/4	1/4	0.61	1/3	2/3	0.72
D/L HbA_{1c} <7.5%	34/52	18/52		20/34	14/34	
D/L HbA_{1c} >7.5%; <8.5%	11/21	10/21		6/11	5/11	
D/L HbA1c >8.5%	38/64	26/64	0.56	17/38	21/38	0.48
D/PR Type 1 DM	9/19	10/19		7/9	2/9	
D/PR Type 2 DM	60/86	26/86	0.10	43/60	17/60	1.00
D/PR DM <10 years	33/54	21/54		26/33	7/33	
D/PR DM >10 years	36/51	15/51	0.41	24/36	12/36	0.19
D/PR neuropathy	7/39	32/39		4/7	3/7	
D/PR retinopathy	23/31	8/31		17/23	6/23	
D/PR nephropathy	16/21	5/21	**<0.0001**	10/16	6/16	0.62
D/PR $HbA1_c$ <7.5%	12/23	11/23		10/12	2/12	
D/PR $HbA1_c$ >7.5%;<8.5%	29/41	12/41		21/29	8/29	
D/PR $HbA1_c$ >8.5%	28/41	13/41	0.29	19/28	9/28	0.60

Data was subdivided according to patient type and duration of DM, long-term diabetic complications and glycaemic control (HbA_{1c})
Statistical analysis was performed using Fisher's exact and Chi square parametric tests
D: Total DM patients; D/L: DM patients from London, UK; D/PR: DM patients from Parma, Italy
* For 36 DM patients from London it was not possible to establish the duration of their disease. Of these, 22 harboured oral yeasts

Table 4.4: *Candida* spp. and genotypic distribution of *C. albicans* isolates in all the carriers

Patients	*C. albicans*	A	B	C	Other species
D/Male	61/78	50	9	2	18**
D/Female	60/74	49	7	4	15**
D/ <60 years old	56/64	46	8	2	10**
D/ >60 years old	65/88	53	9	3	23**
D/dentate	66/75	54	11	1	10**
D/dentures	55/77	45	6	4	23**
D/tobacco users	25/28	19	5	1	5**
D/non-tobacco users	96/124	80	12	4	28**
C/Male	26/36	21	4	1	11***
C/Female	30/37	25	4	1	8***
C/ <60 years old*	38/45	34	3	1	7***
C/ >60 years old*	12/19	9	3	0	7***
C/dentate	35/45	29	5	1	11***
C/dentures	21/28	17	3	1	8***
C/tobacco users	10/17	6	3	1	8***
C/non-tobacco users	46/56	40	5	1	11***
Male total	87/114	71	13	3	29
Female total	90/111	74	11	5	23
<60 years old total	94/109	80	11	3	17
>60 years old total	77/107	62	12	3	30
Dentate total	101/120	83	16	2	21
Dentures total	76/105	62	9	5	31
Tobacco users total	35/45	25	8	2	13
Non-tobacco users total	142/180	120	17	5	39

Data was subdivided according to patient gender, age, denture status and smoking habits
D: DM patients; C: control non-DM subjects
* Of the twelve patients who did not give their date of birth (special needs patients), 10 harboured oral yeasts. Of these ten, 9 yielded one *Candida* strain each and one additional non-DM subject yielded two *Candida* strains
** One hundred and fifty-two DM patients from Parma yielded a single *Candida* strain; 2 additional patients each yielded two strains (154 isolates in total)
*** Seventy-one non-DM control patients yielded a single *Candida* strain; 2 additional control patients each yielded 2 strains (75 isolates in total)

Table 4.5: Oral *Candida* growth and species isolated from the two groups of patients with DM

Candida isolates	Parma DM patients	DM London patients	p value
Growth	69/105 (65.7%)	83/137 (60.6%)	
No growth	36/105 (34.2%)	54/137 (39.4%)	0.42
<100 cfu/ml	50/69 (72.5%)	43/83 (51.8%)	
>100 cfu/ml	19/69 (27.5%)	40/83 (48.2%)	**0.01**
***C. albicans* total**	52/71* (73.2%)	69/83 (83.1%)	
Other species total	19/71* (26.8%)	14/83 (16.9%)	0.16
***C. albicans* A**	40/52 (76.9%)	59/69 (85.5%)	
***C. albicans* B**	10/52 (19.2%)	7/69 (10.1%)	
***C. albicans* C**	2/52 (3.9%)	3/69 (4.4%)	0.47
C. glabrata	7/19 (36.9%)	5/14 (35.8%)	1.00
C. lusitaniae	2/19 (10.5%)	0/14 (0%)	0.49
C. tropicalis	2/19 (10.5%)	1/14 (7.1%)	1.00
C. parapsilosis	7/19 (36.9%)	1/14 (7.1%)	0.09
C. krusei	0/19 (0%)	1/14 (7.1%)	0.42
C. guilliermondii	0/19 (0%)	1/14 (7.1%)	0.42
C. dubliniensis	1/19 (5.2%)	5/14 (35.8%)	0.07

Statistical analysis was performed using Fisher's exact and Chi square parametric tests

Table 4.6: *Candida* spp. and genotypic distribution of *C. albicans* isolates in patients with DM

DM patients	*C. albicans*	A	B	C	Other species
D/ Type 1 DM	36/42	28	7	1	6**
D/Type 2 DM	85/110	71	10	4	27**
D/DM <10 years*	45/55	38	3	4	12**
D/DM >10 years*	60/75	48	11	1	15**
D/neuropathy	25/31	21	4	0	6**
D/retinopathy	32/46	24	7	1	14**
D/nephropathy	15/19	12	3	0	5**
D/HbA_{1c} <7.5%	37/46	30	5	2	10**
D/HbA_{1c} >7.5%; <8.5%	31/40	24	7	0	10**
D/HbA_{1c} >8.5%	53/66	45	5	3	13**
D/L Type 1 DM	28/33	25	3	0	5
D/L Type 2 DM	41/50	34	4	3	9
D/L DM <10 years*	19/22	16	0	3	3
D/L DM >10 years*	34/39	30	4	0	5
D/L neuropathy	20/24	18	2	0	4
D/L retinopathy	18/23	16	2	0	5
D/L nephropathy	2/3	2	0	0	1
D/L HbA1c <7.5%	27/34	21	4	2	7
D/L HbA_{1c} >7.5%; <8.5%	10/11	9	1	0	1
D/L HbA1c >8.5%	32/38	29	2	1	6
D/PR Type 1 DM	8/9	3	4	1	1**
D/PR Type 2 DM	44/60	37	6	1	18**
D/PR DM <10 years	26/33	22	3	1	9**
D/PR DM >10 years	26/36	18	7	1	10**
D/PR neuropathy	5/7	3	2	0	2**
D/PR retinopathy	14/23	8	5	1	9**
D/PR nephropathy	13/16	10	3	0	4***
D/PR $HbA1_c$ <7.5%	10/12	9	1	0	3**
D/PR $HbA1_c$ >7.5%; <8.5%	21/29	15	6	0	9**
D/PR $HbA1_c$ >8.5%	21/28	16	3	2	7**

Data was subdivided according to patient type and duration of DM, long-term diabetic complications and glycaemic control ($HbA1_c$)

D: Total DM patients; D/L: DM patients from London, UK; D/PR: DM patients from Parma, Italy

* For 36 of DM patients from London it was not possible to establish the duration of their disease. Of these 22, harboured oral yeasts

** Of the 152 DM patients who harboured oral yeasts, 69 from Parma yielded a single *Candida* strain; 2 additional DM patients from Parma each yielded two strains (154 isolates in total)

Table 4.7: Oral *Candida* isolation and load (cfu/ml) in the two different groups of patients with DM

Patients	Growth	No growth	p value	<100 cfu/ml	>100 cfu/ml	p value
D/L Male	43/70	27/70		22/43	21/43	
D/L Female	40/67	27/67	0.86	21/40	19/40	1.00
D/L <60 years old	44/78	34/78		27/44	17/44	
D/L >60 years old	39/59	20/59	0.29	16/39	23/39	0.08
D/L dentate	54/97	43/97		33/54	21/54	
D/L dentures	29/40	11/40	0.08	10/29	19/29	**0.02**
D/L tobacco users	13/19	6/19		5/13	8/13	
D/L non-tobacco users	70/118	48/118	0.61	38/70	32/70	0.37
D/PR Male	35/55	20/55		26/35	9/35	
D/PR Female	34/50	16/50	0.68	24/34	10/34	0.79
D/PR <60 years old	20/35	15/35		18/20	2/20	
D/PR >60 years old	49/70	21/70	0.19	32/49	17/49	**0.04**
D/PR dentate	21/45	24/45		16/21	5/21	
D/PR dentures	48/60	12/60	**0.0008**	34/48	14/48	0.77
D/PR tobacco users	15/20	5/20		10/15	5/15	
D/PR non tobacco users	54/85	31/85	0.43	40/54	14/54	0.74

Data was subdivided according to patient gender, age, denture status and smoking habits
Statistical analysis was performed using Fisher's exact and Chi square parametric tests
D/L: DM patients from London, UK; D/PR: DM patients from Parma, Italy

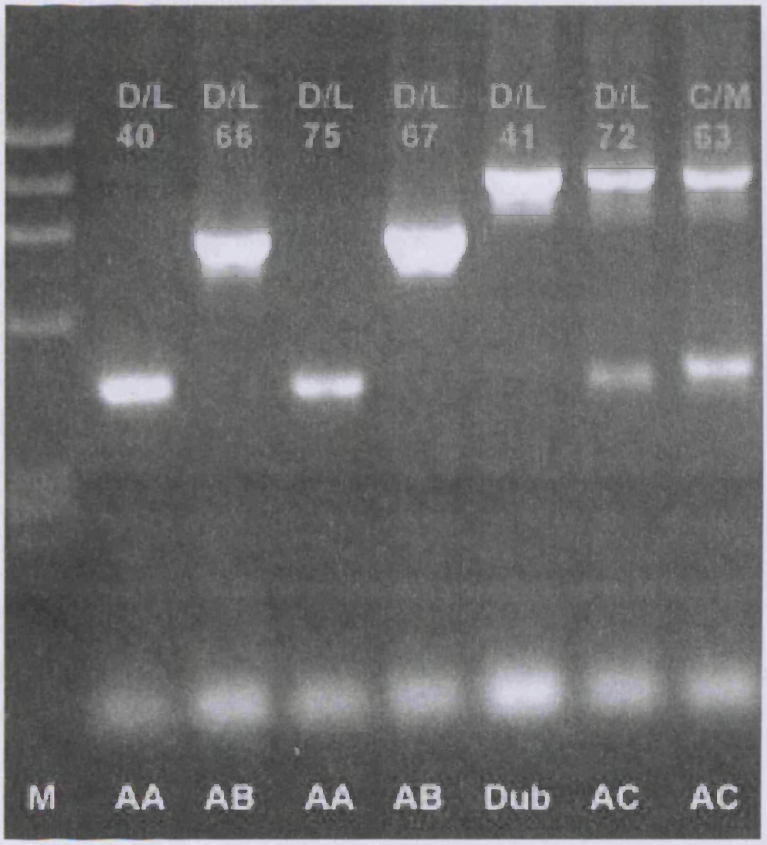

Figure 4.1: Example of electrophoretic separation of PCR products of the INT region of *Candida* isolates from patients with and without DM

M: marker; AA: *C. albicans* A; AB: *C. albicans* B; AC: *C. albicans* C; Dub: *C. dubliniensis*

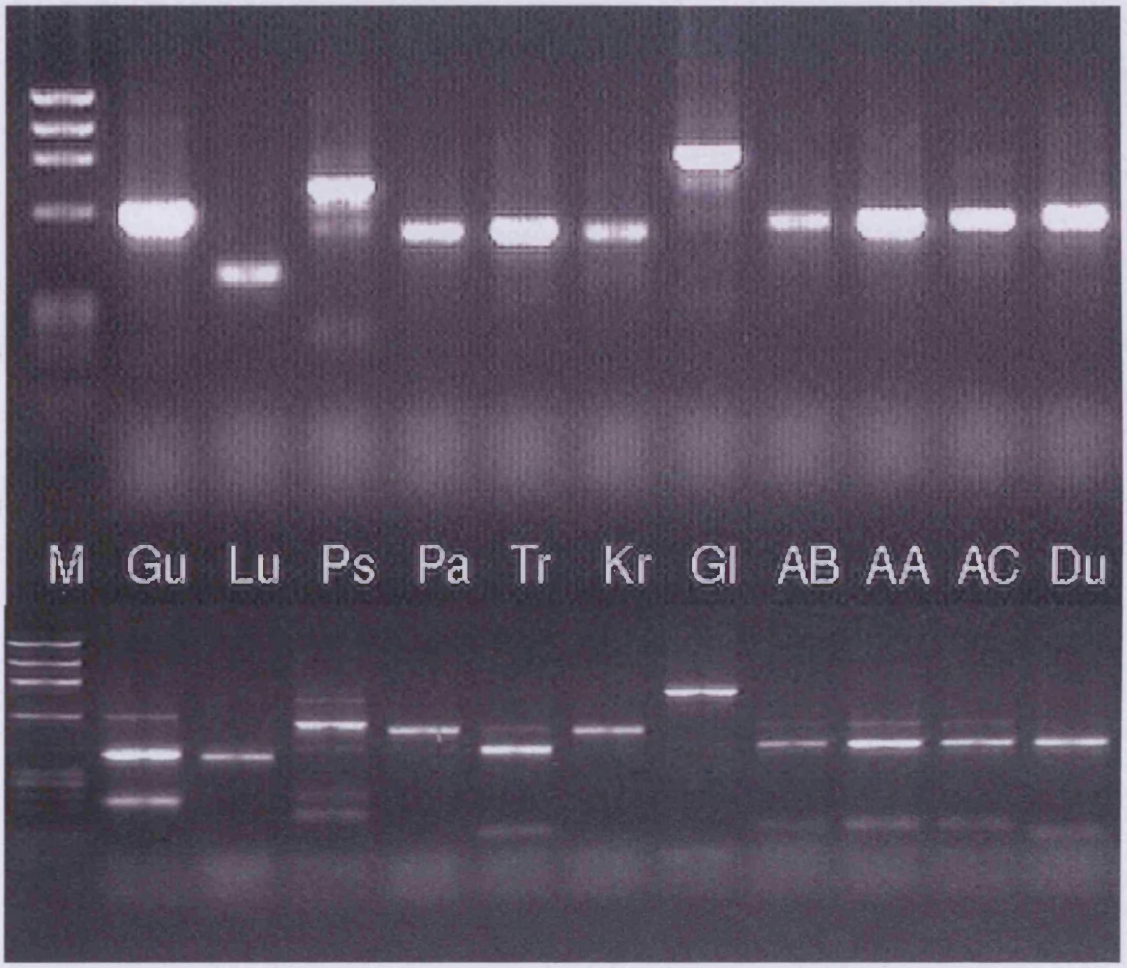

Figure 4.2: *Dde* I undigested and digested PCR products of the ITS region

Dde I undigested products (upper) and digested products (lower). M: Marker; Gu: *C. guilliermondii*; Lu: *C. lusitaniae*; Ps: *C. kefyr* (once *C. pseudotropicalis*); Pa: *C. parapsilosis*; Tr: *C. tropicalis*; Kr: *C. krusei*; Gl: *C. glabrata*; AB: *C. albicans* B; AA: *C. albicans* A; AC: *C. albicans* C; Du: *C. dubliniensis*

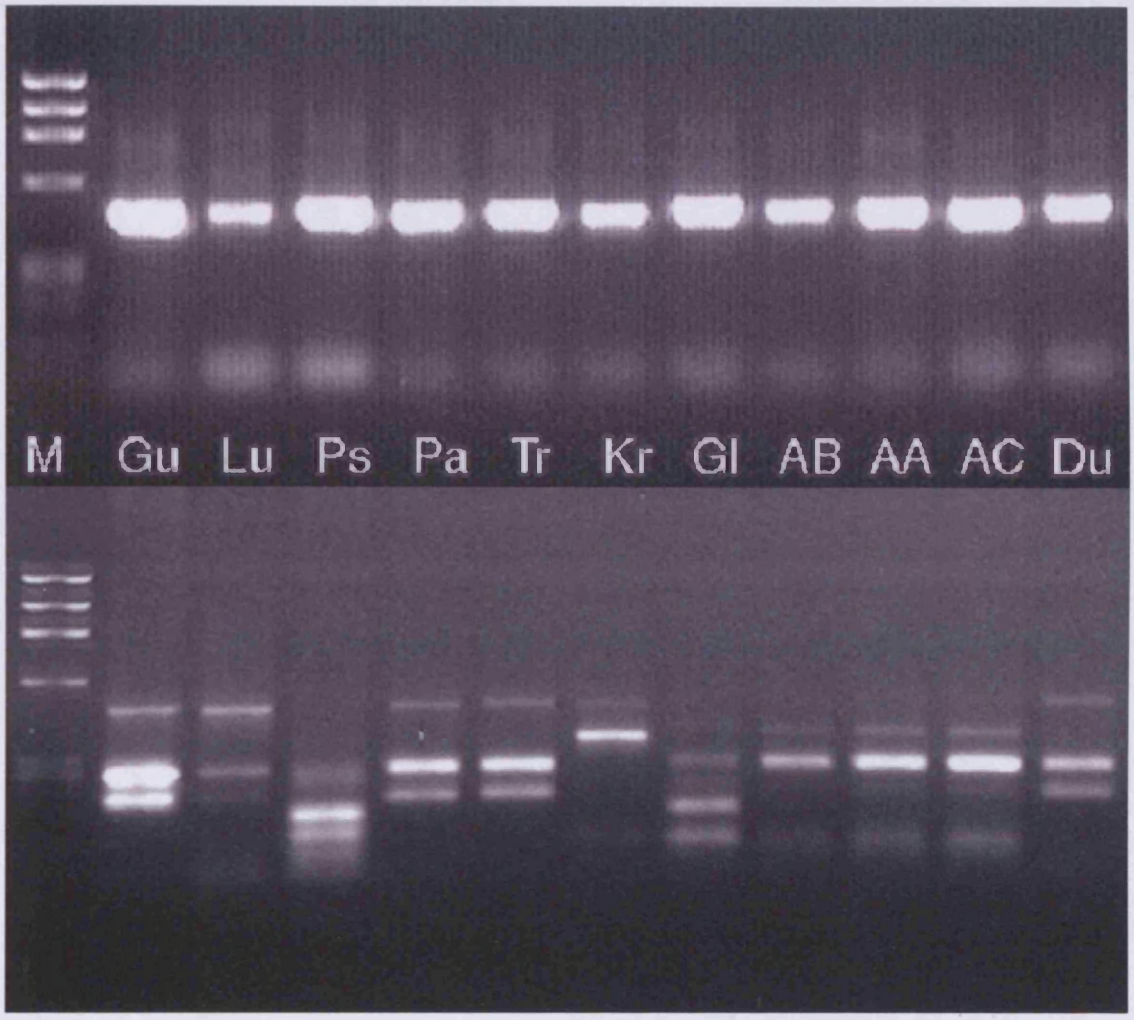

Figure 4.3: *Hae* III undigested and digested PCR products of the V3 25S rDNA region

Hae III undigested (upper) and digested (lower) PCR products. M: Marker; Gu: *C. guilliermondii*; Lu: *C. lusitaniae*; Ps: *C. kefyr* (previously termed *C. pseudotropicalis*); Pa: *C. parapsilosis*; Tr: *C. tropicalis*; Kr: *C. krusei*; Gl: *C. glabrata*; AB: *C. albicans* B; AA: *C. albicans* A; AC: *C. albicans* C; Du: *C. dubliniensis*

CHAPTER 5

Virulence attributes of oral yeasts

5.1 Introduction

Candida species, like all pathogenic microorganisms, have developed specific virulence mechanisms that confer the ability to colonize host surfaces, invade deeper host tissue or evade host defences (Hube & Naglik, 2002).

Adherence to host tissue is the first step in the pathogenic process: once initial contact between host tissues and *Candida* has occurred, enzymes facilitate adherence by damaging or degrading cell membranes and extracellular proteins (Cotter & Kavanagh, 2000). The adherence mechanisms of *Candida* species to many cell types or surfaces are complex and still not elucidated. Adherence is achieved by a combination of specific (ligand-receptor interactions) and non-specific mechanisms (electrostatic forces, aggregation, and cell-surface hydrophobicity) (Cotter & Kavanagh, 2000).

Specific adherence is mediated by a number of target proteins located in the epithelial cell surface or in the subepithelial extracellular matrix (ECM). Of these, fibronectins are adhesive glycoproteins with a high molecular weight involved in cell adhesion and cell migration, and located in the ECM interstitium (Castellani *et al*, 1986; Cotter & Kavanagh, 2000). Other microorganisms apart from *Candida*, such as *Staphylococcus aureus* and *Streptococcus pyogenes*, have also been shown to adhere to ECM protein fibronectins *in vitro* (Skerl *et al*, 1984).

Furthermore, molecules (transmembrane integrins) present on the surface of *C. albicans* are thought to mediate adherence to ECM molecules (Hostetter, 1994). These "adhesins" function by recognising ligands that contain amino-acid sequences

such as the Arg-Gly-Asp (RGD) sequence (Varner & Cheresh, 1996). *Candida albicans* binds RGD-containing proteins, such as fibronectins, laminin and collagen types I and IV, through integrin-related structures (Klotz *et al*, 1994).

The complex pathogenesis of *Candida* species is aided by the production of a range of extracellular enzymes that facilitate adherence and/or tissue penetration. The three most significant hydrolytic enzymes produced by *Candida* species, and most notably by *C. albicans*, are secreted aspartic proteinases (Saps), phospholipases and lipases (Hube & Naglik, 2002).

Many *Candida* species, including *C. albicans*, *C. dubliniensis* (Gilfillan *et al*, 1998), *C. tropicalis* (Togni *et al*, 1991; Monod *et al*, 1994; Zaugg *et al*, 2001) and *C. parapsilosis* (de Viragh *et al*, 1993; Monod *et al*, 1994) are known to possess SAP genes and produce active extracellular proteinases *in vitro*. However, less pathogenic or non-pathogenic *Candida* species do not produce significant levels of proteinases, and this has led to the hypothesis that fungal pathogenicity is directly linked to the extent of Sap production.

Studies show that the targets of the proteolytic attack of Saps, *in vitro* and *in vivo*, are very different. Extracellular matrix and host surface proteins, such as laminin, fibronectin, and mucin are efficiently degraded by Saps, in particular by Sap 2, the most active Sap *in vitro*, aiding yeast-cell adhesion to buccal epithelial cells. Research has shown that Sap 2 also hydrolyses secretory IgA, normally resistant to most bacterial proteinases (Naglik *et al*, 2003). Sap 2 activity is optimal at low pH (2.5-5.5) (Naglik *et al*, 2003; Naglik *et al*, 2004), but it is also stable at neutral pH and is active under these conditions (Capobianco *et al*, 1992; Wagner *et al*, 1995). Sap 4 and Sap6 are extremely active at physiological pH (Hube & Naglik, 2002). This stability of Saps at different pH ranges (2.0-7.0) may assist *Candida* species, in

particular *C. albicans*, to colonize and/or infect hosts in a neutral pH environment, such as the oral cavity.

Different classes of phospholipases are known and classified according to their mode of action (Hube & Naglik, 2002). In *C. albicans,* phospholipases A, B, C and lysophospholipases may damage host-cell membranes and facilitate tissue invasion. Recently, it has also been shown that non-*C. albicans* species secrete phospholipases at lower levels (Ghannoum, 2000) compared with *C. albicans*. Furthermore, a correlation between phospholipase activity *in vitro* and virulence has also been demonstrated (Ghannoum, 2000; Hube & Naglik, 2002).

There are few studies which have investigated the expression of both the adhesion and extracellular proteinase production by oral *Candida* isolates from patients with DM. As reported in Chapter 1 (Section 1.8), DM is a common disease found worldwide and it has been previously suggested that oral candidal infections may be more frequent or severe in patients with DM (Aly *et al*, 1992; Dorocka-Bobkowska *et al*, 1996; Willis *et al*, 1999). However, it has been recently reported that only a small number of patients affected by DM develop oral candidosis (Section 1.8.9.1.1-3.3.1) (Manfredi *et al*, 2002). Systemic (*e.g.* the degree of glycaemic control) or local factors (e.g. presence of dentures) may influence the balance between the host and yeasts, and favour the transformation of *Candida* isolates from commensal to pathogenic microorganisms. It would be interesting to investigate whether there are virulent attributes, such as adhesion or proteinase expression, whose activity is enhanced for isolates from DM patients.

Aim: The aim of this study was to evaluate the *in vitro* extracellular proteinase production and the ability of *Candida* isolates to adhere *in vitro* to fibronectin. A

comparison of these traits was made for isolates from two geographically different groups of DM patients and from a healthy control group of non-DM subjects.

Hypothesis: That there are differences in the ability of *Candida* recovered from the oral cavities of DM and non DM patients to adhere to fibronectin or produce extracellular proteinase.

5.2 Methods

Oral yeasts isolated from each group of patients were evaluated using specific adhesion (Section 2.6) and proteinase (Section 2.7) assays. Adhesion was assessed by quantifying the attachment of *Candida* to paramagnetic beads (Dynabeads, Dynal) coated primarily with fibronectin and secondarily with BSA. Results were expressed as the number of *Candida* cells bound to a dynabead. Proteinase activity of *Candida* was assessed using a spectrophotometric method (Section 2.7) and results were expressed as a ratio between the spectrophotometer reading (280 nm) and numbers of cells/ml.

The distribution of the proteinase and adhesion activity for these isolates failed the Kolmogorov - Smirnov test for statistical analysis normality. Statistical parametric tests assume that the evaluated data is distributed according to an ideal Gaussian distribution. Since the Gaussian distribution is also referred to as the Normal distribution, the test is also called a normality test. The Kolmogorov – Smirnov test quantifies discrepancies between the distribution of data and an ideal Gaussian distribution - a larger value denotes larger discrepancies (GraphPad Software Inc. - www.graphpad.com).

In this chapter, data was not distributed according to the normality test conditions and parametric tests were not applicable. In addition, the shape distribution of data

obtained for both adhesion and proteinase of the *Candida* isolates of the groups studied was very different and anomalous values (outliers) were present in each group (DM and non-DM). As a consequence, all the isolates (isolated from DM patients from London, UK, Parma, Italy and from non-DM subjects) were divided above and below the median for each of these respective tests (median for adhesion: 116.38; median for proteinase: 0.153442), and each isolate was thus classified as having either high or low extra-cellular proteinase activity and high or low adhesive properties. The differences among or between groups were evaluated using Fisher's exact test or Chi square test and values were deemed significant when the probability (p) was less than or equal to 0.05.

5.3 Isolates

The 229 *Candida* strains isolated from the oral cavity of the two groups of DM patients (83 from the 137 UK DM patients and 71 isolates from the 105 Italian patients, 154 in total) and the non-DM subjects (75 out of the 130 non-DM subjects) were examined in this study.

5.4 Results

5.4.1 Adhesion and proteinase activity of oral *Candida* isolates of patients with and without DM

Analysis of adherence and proteinase production by the 154 *Candida* isolates from the DM patients demonstrated that there were no significant associations with age, gender, smoking habits and denture status (Table 5.1).

However, *Candida* from DM patients with lower levels of oral yeast colonisation (<100 cfu/ml) had higher adhesion levels (p=0.01) than isolates from DM patients with a high oral candidal load (>100 cfu/ml) (Table 5.1).

No statistical difference was found between *Candida* spp. or genotypic distribution of *C. albicans* and adhesion or proteinase expression ($p>0.05$). Furthermore, although the type of DM, duration of disease, long-term diabetic complications and haemoglobin glycosylation levels did not influence the adhesion ($p>0.05$) of the *Candida* spp. isolated from the DM patients, significantly higher levels of proteinase ($p=0.02$) were detected for isolates recovered from type 2 DM patients compared with type 1 DM patients (Table 5.1).

The *in vitro* ability of 75 *Candida* strains from non-DM subjects to adhere and produce SAPs was not significantly associated with patient age, gender, smoking habits, denture status, oral candidal load (cfu/ml), species of *Candida* or the genotypic distribution of *C. albicans* ($p>0.05$).

A comparison of the adhesion levels of *Candida* isolates from the DM patient population with those of the non-DM subjects showed that there was significantly higher levels of adhesion for the yeast from the DM patient groups ($p<0.0001$) (Table 5.2). More specifically, the *in vitro* ability to adhere was found to be significantly higher for *Candida* isolates from patients with DM than those isolated from non-DM controls for all the variables evaluated.

No significant differences in *Candida* proteinase production were observed between DM and non-DM subjects ($p=0.48$). However, higher levels of proteinase were found for isolates from non-DM control subjects with a lower degree of colonisation (<100 cfu/ml) compared with isolates from the DM patients ($p=0.03$) (Table 5.2).

5.4.2 Adherence and proteinase activity of all *Candida* isolates

Analysis of all 229 *Candida* isolates from patients with DM and healthy subjects indicated that the adherence of isolates from individuals with levels of oral yeast colonisation <100 cfu/ml was significantly higher ($p=0.0006$) than other individuals

(Table 5.3). No other differences ($p>0.05$) were observed in adherence or proteinase production of isolates from any of the 229 *Candida* carriers evaluated in this study (Table 5.3).

5.4.3 Influence of geographical locale on adhesion and proteinase activity of *Candida* isolates of patients with DM

Significant differences were observed between the two geographically different DM populations in terms of the isolates' ability to adhere or produce proteinase (Table 5.4a and 5.4b). There was a tendency ($p=0.06$) for oral isolates from patients with DM from Italy to have higher adherence than isolates from UK DM patients and also for the latter to produce higher levels of extracellular proteinase ($p=0.053$).

Adherence of *Candida* isolates from Italian DM patients who wore dentures was significantly higher ($p=0.03$) than those of isolates from UK DM patients. Furthermore, oral yeasts isolated from Italian DM patients whose diabetic control was good ($HbA1_c<7.5\%$) exhibited higher adherence ($p=0.01$) than isolates from London patients with the same level of glycaemic control (Table 5.4b).

Higher levels of proteinase activity was found in *C. albicans* isolates from UK DM patients ($p=0.04$), particularly with *C. albicans* genotype A ($p=0.04$), compared with isolates from Italian DM patients (Table 5.4a).

Analyses of proteinase activity of isolates from tobacco users showed that *Candida* from UK DM patients expressed higher proteinase levels ($p=0.02$) than those from patients with DM from Italy (Table 5.4a).

Finally, higher levels of proteinase activity ($p=0.02$) was observed for the oral isolates from UK DM patients with good metabolic control ($HbA1_c<7.5\%$) compared with isolates from Italian DM patients with the same level of glycaemic control (Table 5.4b).

5.5 Discussion

This study has investigated the expression of two important virulence attributes of *Candida* isolates recovered from DM patients and healthy control subjects. There are few studies that have investigated these two pathogenic factors for oral isolates of DM patients and in general, these have not examined individuals with a spectrum of DM. In addition, researchers have not simultaneously examined more than one virulence attribute (Darwazeh *et al*, 1990; Willis *et al*, 2000a).

In the present study, comparison of the adherence of the 154 *Candida* isolates from DM patients and non-DM subjects, revealed significant differences in the *in vitro* adhesion between these two populations. In particular, isolates from DM patients exhibited a higher capacity to adhere to fibronectin ($p<0.0001$) compared with non-DM controls.

The adherence assay used was based on the binding of yeasts to fibronectin. This is one of the ECM glycoproteins and it has recently been identified as a *Candida* adhesin-receptor (Ala1p) (Gaur & Klotz, 1997; Gaur *et al*, 1999; Gaur *et al*, 2002). The adherence assay used in this study was based on the interaction between fibronectin and *Candida* and therefore provided a general indicator to overall adherence potential. Fibronectin was selected as a marker for adherence as the role of fibronectin-*Candida* interactions in the adherence process had already been established (Yan *et al*, 1996; Gaur & Klotz, 1997; Cotter *et al*, 1998; Gozalbo *et al*, 1998; Senet, 1998; Yan *et al*, 1998a; Yan *et al*, 1998b; Rodier *et al*, 1999; Pendrak *et al*, 2000; Singleton *et al*, 2001; Rauceo *et al*, 2004). A number of different proteins (*e.g.* keratin, involucrin, salivary mucins) that may be involved in surface interaction may also have been used, but these assays were considered to be more suitable for evaluating the molecular adherence mechanism of *Candida* to epithelial surfaces, rather than variability of the yeast in adherence.

The reason for the enhanced adhesion of isolates of patients with DM may reflect the presence of a higher concentration of sucrose/glucose in the saliva of patients affected by DM compared to healthy non-DM subjects. It has been observed that yeast growth and adhesion may be enhanced by high blood and saliva glucose levels (Odds *et al*, 1978; Samaranayake *et al*, 1984a), which can serve as nutrients for *Candida* cells. It could therefore be surmised that strains isolated from DM patients with higher glucose salivary levels, may have an inherently higher ability to adhere due to the selection of such strains in the high sugar oral environment associated with DM.

Although this study did not show that DM patients with lower levels of glycaemic control had higher levels of oral *Candida* carriage or yeast with higher adhesion levels, the strains isolated from the oral cavity of patients suffering from DM may have, however, increased their ability to adhere *in vitro*. It is likely that more than one factor may promote *Candida* carriage and enhance the isolates' adhesion activity in DM patients.

Analysis of proteinase production by *Candida* isolates from DM and non-DM subjects did not reveal any significant differences ($p=0.48$). However, significant association was observed within the DM patient group. Interestingly, it was evident from the present study that yeast isolated from type 2 DM patients had significantly higher expression of extracellular proteinases ($p=0.02$) compared with isolates from type 1 DM patients. This finding may well reflect possible variation (related to DM type) in the levels of certain salivary constituents such as secretory IgA, salivary glucose or enzyme levels *e.g.* matrix metalloproteinase (MMP-8) and gelatinase (MMP-9) (Collin *et al*, 2000b). Indeed it has previously been reported (McCullough *et al*, 1995) that some oral infections such as periodontitis in type 2 DM patients is related to elevated salivary MMP-8 levels. In addition, salivary lysozyme levels are known to be influenced by DM (Stevens *et al*, 1990), and could theoretically effect

SAP activity (Rayfield *et al*, 1982; Scherer & Stevens, 1987) and thus extracellular proteinase *in vitro* production, although this remains unproven.

Although many of the pathogenetic *Candida* spp. have been shown to possess SAP genes and produce active extracellular proteinases *in vitro*, it has also been reported that the most virulent species, such as *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis*, produce more proteinases *in vitro* than the less virulent species (Ruchel, 1992; Naglik *et al*, 2003; Naglik *et al*, 2004). Interestingly, no differences were observed in the proteinase production between *C. albicans* and non-*C. albicans* spp. in the present study. This result may be due to the difference in the number of such species between the two groups (as there were many more *C. albicans* than non-*C. albicans* isolates). It has however, been reported that even if *C. albicans* exhibits higher *in vitro* Sap activity, such differences may partly be due, to assay sensitivity (Naglik *et al*, 2003). Thus it would be of interest to evaluate proteinase production of the isolates studied with different relevant assays, in order to confirm (or not) the present result.

Recently, it has been reported that *C. albicans* genotype B produces significantly higher proteinase and phospholipase activity compared to genotypes A or C (Sugita *et al*, 2002). In this present study, no differences in extracellular proteinase production and *C. albicans* genotype were observed when the isolates from all the carriers were evaluated. However, *C. albicans* genotype A isolated from London DM patients expressed higher levels of extracellular proteinase than those from Italian DM patients (Table 5.4). Further research is however necessary in order to elucidate the existence of any relationship between Saps and/or phospholipase activity and the *C. albicans* genotype.

Isolates from DM patients carrying a lower number of oral yeasts (<100 cfu/ml) had expressed greater adhesion ($p=0.01$) than isolates from patients who had a higher

number of oral yeasts (Table 5.1). This was also observed for isolates from all the *Candida* carriers evaluated in this study (Table 5.3).

The inverse relationship between the degree of colonisation and the ability of isolates to adhere is intriguing. The assay used to evaluate adhesiveness directly measured the isolates' ability to adhere to fibronectin. Therefore, it may be assumed that, as the number of yeasts present in the oral cavity increases, interaction between yeasts themselves becomes of greater importance than interaction between the yeast and the host, as was analysed in this study. Such a theory supports the concept of biofilm formation (Baillie & Douglas, 1998) and the increasing importance of interaction between microorganisms (Sullivan & Coleman, 1997; Willis *et al*, 2000b). The results of the previous Chapter, where it was established that DM did not significantly increase the carriage of oral yeasts. The results of the present Chapter show no evidence correlating oral yeasts *in vitro* virulence and colonisation in DM patient. Thus, in DM, neither oral yeast number nor phenotype are unique, further support the notion that patients with DM, at least when treated, are not at increased risk of developing oral candidosis.

Further research could be directed at examining agglutinin-like sequence genes (ALS), a family of at least 9 genes that encode cell surface glycoproteins, mainly characterised in *C. albicans* and related *Candida* spp. (Hoyer *et al*, 1995; Hoyer *et al*, 1998; Chandra *et al*, 2001; Hoyer, 2001; Hoyer & Hecht, 2001) and are known to vary in expression depending upon environmental conditions.

The presence of oral prostheses may influence the capability of oral yeasts to adhere. In this study, particularly high levels of adhesion were observed in *Candida* isolates from Italian DM patients who wore dentures (Table 5.2 and 5.4a).

It is well known that the presence of oral prostheses such as dentures and removable (or fixed) orthodontic appliances can increase oral yeast carriage. The denture may function as a chronic reservoir of infection and dissemination (Davenport, 1970; Verran & Maryan, 1997; Maza *et al*, 2002), and surface irregularities would increase the likelihood of microorganisms remaining on the surface after the prosthesis has been cleaned.

It may be that DM, rather than the sole presence of a dental prosthesis, affects adherence, as the adherence of isolates from the control subgroup was not influenced by the presence of dentures. Furthermore, it has been reported (Radford *et al*, 1999) that *in vivo*, microorganisms never attach directly to prostheses surfaces, since they are always covered with a salivary pellicle of various degrees of maturity. It may be that any enhanced interactions between yeasts and local factors such as a low salivary content (due to the presence of dentures and hypoglycaemic agents) or poor metabolic control may increase the ability of *Candida* to adhere to oral mucosa, rather than any yeast-derived factor.

It is known that adhesion is strongly related to the presence of specific sugars (Samaranayake & MacFarlane, 1980; Samaranayake *et al*, 1980; McCourtie & Douglas, 1981) and that the adherence of *C. albicans* to acrylic surfaces increases when cultured in various carbon sources, including glucose, maltose and galactose (McCourtie & Douglas, 1984). This increase has been attributed to enhanced formation of a fibrillar-floccular layer, demonstrated by ruthenium red or polycationic ferritin staining, which indicates that this layer may be rich in acidic polysaccharides (McCourtie & Douglas, 1981).

This study has investigated the influence of DM upon the ability of oral *Candida* isolates to enhance the expression of two of the most important virulence attributes of

Candida spp.: adhesion and production of extracellular proteinase. The assay used in this study showed that although *Candida* from DM patients had a greater ability to adhere compared with those from healthy subjects, higher adhesion levels were shown in Italian patients with DM wearing dentures than in UK patients with DM and wearing dentures. In addition, higher levels of adhesion were also recorded in patients with a lower oral *Candida* colonisation (< 100 cfu/ml).

In vitro extracellular proteinase expression was not significantly higher in *Candida* isolates of patients with DM compared to those of healthy subjects. However, higher extracellular proteinase levels were observed in isolates from type 2 DM patients when compared with type 1 DM patients.

As reported, it would be interesting to correlate the ALS and SAP gene expression obtained by RT-PCR for *Candida* isolates grown in the culture media previously used in the adhesion and extracellular proteinase production assays.

Table 5.1: Ability to adhere and extracellular proteinase production obtained from *Candida* isolates of patients with DM

DM patients	Adhesion above median	Adhesion below median	p value	Proteinase above median	Proteinase below median	p value
Male	45	34	0.18	41	38	0.33
Female	51	24		33	42	
<60 years	41	25	1.00	33	33	0.74
>60 years	55	33		41	47	
Tobacco users	20	10	0.67	15	15	0.84
Non-tobacco users	76	48		59	65	
Dentate	46	30	0.73	39	37	0.51
Dentures	50	28		35	43	
<100 cfu/ml	67	28	**0.01**	40	55	0.06
>100 cfu/ml	29	30		34	25	
C. albicans	73	48	0.41	60	61	0.55
Non-*C. albicans*	23	10		14	19	
***C. albicans* A**	58	41	0.84	52	47	0.27
***C. albicans* B**	12	5		7	10	
***C. albicans* C**	3	2		1	4	
Type 1 DM	26	16	1.00	27	15	**0.02**
Type 2 DM	69	43		49	63	
<10 years DM*	34	23	0.85	24	33	0.15
>10 years DM*	46	29		42	33	
$HbA1_c$ <7.5%	30	17	0.84	24	23	0.79
$HbA1_c$ >7.5%;<8.5%	24	17		18	23	
$HbA1_c$ >8.5%	42	24		32	34	
Neuropathy	19	12	0.36	17	14	0.44
Retinopathy	26	20		26	20	
Nephropathy	15	5		8	12	

Statistical analysis was performed sub-classifying the values above and below the median of each assay (adhesion: 116.38 and proteinase: 0.153442), then Fisher's exact or Chi square tests were used to analyse data obtained

* For 36 DM patients from London it was not possible to establish the duration of their disease. Of these, 22 harboured oral yeasts

Table 5.2: Ability to adhere and extracellular proteinase production of *Candida* isolates of patients with DM compared with non-DM control subjects

Patients	Adhesion above median	Adhesion below median	p value	Proteinase above median	Proteinase below median	p value
Male						
D	45	34	**0.003**	41	38	0.42
ND	9	28		16	21	
Female						
D	51	24	**<0.0001**	33	42	0.07
ND	10	28		24	14	
<60 years*						
D	41	25	**0.0001**	33	33	0.44
ND	11	34		26	19	
>60 years*						
D	55	33	**0.02**	41	47	1.00
ND	6	13		9	10	
Tobacco users						
D	20	10	**0.001**	15	15	0.55
ND	3	15		11	7	
Non-tobacco users						
D	76	48	**<0.0001**	59	65	0.74
ND	16	41		29	28	
<100 cfu/ml						
D	67	28	**<0.0001**	40	55	**0.03**
ND	6	18		16	8	
>100 cfu/ml						
D	29	30	**0.01**	34	25	0.33
ND	13	38		24	27	
Dentate						
D	46	30	**0.001**	39	37	0.70
ND	14	32		26	20	
Dentures						
D	50	28	**<0.0001**	35	43	0.82
ND	5	24		14	15	
C. albicans						
D	73	48	**<0.0001**	60	61	0.41
ND	14	42		32	24	
Non-*C. albicans*						
D	23	10	**0.03**	14	19	1.00
ND	5	14		8	11	
***C. albicans* A**						
D	58	41	**<0.0001**	52	47	0.37
ND	10	36		28	18	
***C. albicans* B**						
D	12	5	0.08	7	10	1.00
ND	2	6		3	5	
***C. albicans* C**						
D	3	2	1.00	1	4	1.00
ND	1	1		1	1	

Statistical analysis was performed sub-classifying the values above and below the median of each assay (adhesion: 116.38 and proteinase: 0.153442), then Fisher's exact or Chi square tests were used to analyse data obtained
D: DM patients; ND: non-DM control subjects
* Of the twelve non-DM control subjects who did not give their date of birth (special needs patients), 10 harboured oral yeasts. Of these ten, 9 yielded one *Candida* strain each and one additional non-DM subject yielded two *Candida* strains

Table 5.3: Ability to adhere and extracellular proteinase production obtained from all the *Candida* isolate carriers

All carriers	Adhesion above median	Adhesion below median	p value	Proteinase above median	Proteinase below median	p value
Male	54	62	0.29	56	60	0.79
Female	61	52		57	56	
<60 years*	52	59	0.13	59	52	0.41
>60 years*	61	46		50	57	
Tobacco users	23	25	0.74	27	21	0.41
Non-tobacco users	92	89		88	93	
Dentate	60	62	0.79	63	59	0.42
Dentures	55	52		49	58	
<100 cfu/ml	73	46	**0.0006**	56	63	0.42
>100 cfu/ml	42	68		58	52	
C. albicans	87	90	0.63	93	84	0.21
Non-*C. albicans*	28	24		22	30	
***C. albicans* A**	68	77	0.63	80	65	0.16
***C. albicans* B**	14	11		10	15	
***C. albicans* C**	4	3		2	5	

Statistical analysis was performed sub-classifying the values above and below the median of each assay (adhesion: 116.8 and proteinase: 0.153442), then Fisher's exact or Chi square tests were used to analyse data obtained

* Of the twelve non-DM control subjects who did not give their date of birth (special needs patients), 10 harboured oral yeasts. Of these ten, 9 yielded one *Candida* strain each and one additional non-DM subject yielded two *Candida* strains

Table 5.4 a: Ability to adhere and extracellular proteinase production of *Candida* isolates of patients with DM from two different geographic locales

DM patients	Adhesion above median	Adhesion below median	p value	Proteinase above median	Proteinase below median	p value
Male						
D/L	20	23	0.15	26	17	0.11
D/PR	25	11		15	21	
Female						
D/L	26	14	0.62	20	20	0.35
D/PR	25	10		13	22	
<60 years						
D/L	26	18	0.13	24	20	0.43
D/PR	15	7		9	13	
>60 years						
D/L	20	19	0.07	22	17	0.13
D/PR	35	14		19	30	
Tobacco users						
D/L	6	7	0.056	10	3	**0.02**
D/PR	14	3		5	12	
Non-tobacco users						
D/L	40	30	0.35	36	34	0.36
D/PR	36	18		23	31	
Dentate						
D/L	32	22	0.79	31	23	0.13
D/PR	14	8		8	14	
Dentures						
D/L	14	15	**0.03**	15	14	0.48
D/PR	36	13		20	29	
<100 cfu/ml						
D/L	28	15	0.36	22	21	0.14
D/PR	39	13		18	34	
>100 cfu/ml						
D/L	18	22	0.41	24	16	0.77
D/PR	11	8		10	9	
C. albicans						
D/L	37	32	0.09	40	29	**0.04**
D/PR	36	16		20	32	
Non-*C. albicans*						
D/L	9	5	0.70	6	8	1.00
D/PR	14	5		8	11	
***C. albicans* A**						
D/L	30	29	0.06	36	23	**0.04**
D/PR	28	12		16	24	
***C. albicans* B**						
D/L	6	1	0.33	3	4	1.00
D/PR	6	4		4	6	
***C. albicans* C**						
D/L	1	2	0.4	1	2	1.00
D/PR	2	0		0	2	

Statistical analysis was performed sub-classifying the values above and below the median of each assay (adhesion: 116.38 and proteinase: 0.153442), then we used Fisher's exact or Chi square tests

Table 5.4 b: Ability to adhere and extracellular proteinase production of *Candida* isolates of patients with DM from two different geographic locales

DM patients	Adhesion above median	Adhesion below median	p value	Proteinase above median	Proteinase below median	p value
Type 1 DM						
D/L	19	14	0.44	22	11	0.69
D/PR	7	2		5	4	
Type 2 DM						
D/L	27	23	0.17	26	24	0.12
D/PR	42	20		23	39	
DM <10 years*						
D/L	10	12	0.10	13	9	0.43
D/PR	24	11		11	24	
DM >10 years*						
D/L	20	19	0.09	26	13	0.06
D/PR	26	10		16	20	
$HbA1_c$ <7.5%						
D/L	18	16	**0.01**	21	13	**0.02**
D/PR	12	1		3	10	
$HbA1_c$ >7.5% ;<8.5%						
D/L	6	5	1.00	5	6	1.00
D/PR	18	12		13	17	
$HbA1_c$ >8.5%						
D/L	22	16	0.30	20	18	0.45
D/PR	20	8		12	16	
Neuropathy						
D/L	13	11	0.20	12	12	0.41
D/PR	6	1		5	2	
Retinopathy						
D/L	10	13	0.13	14	9	0.76
D/PR	16	7		12	11	
Nephropathy						
D/L	1	2	0.14	0	3	0.24
D/PR	14	3		8	9	

Statistical analysis was performed sub-classifying the values above and below the median of each assay (adhesion: 116.38 and proteinase: 0.153442), then Fisher's exact or Chi square tests were used to analyse data obtained.
D/L: DM patients from London; UK; D/PR: DM patients from Parma, Italy
* For 36 DM patients from London it was not possible to establish the duration of their disease. Of these, 22 harboured oral yeasts

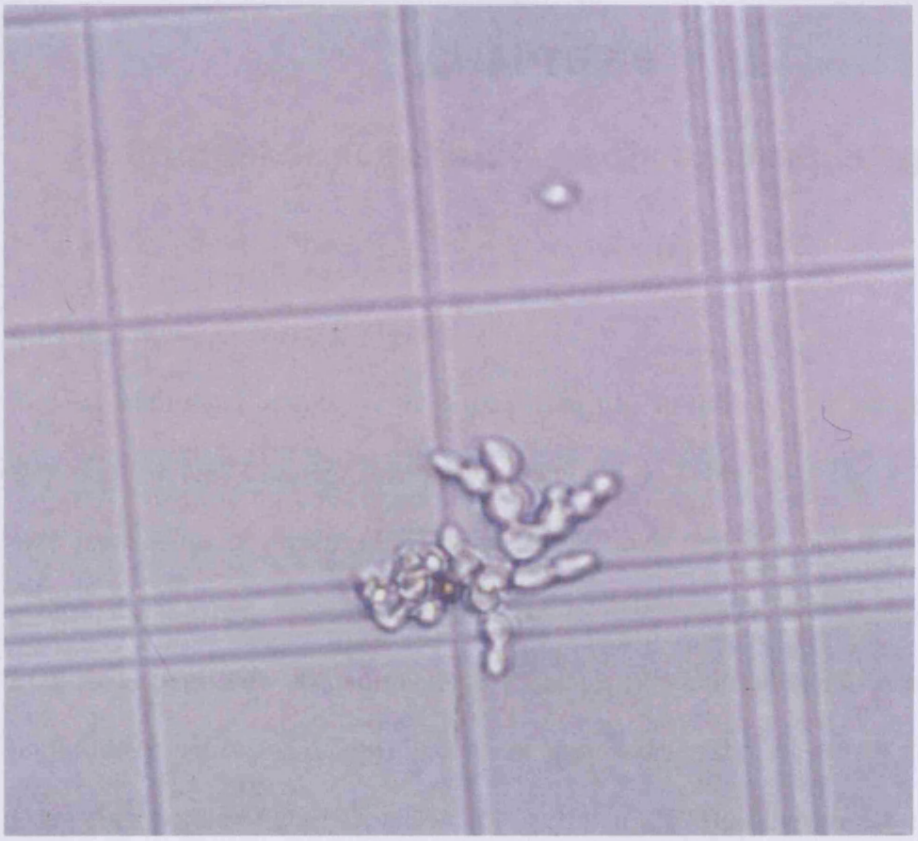

Figure 5.1: Example of aggregation of *Candida* cells to a dynabead and consequent cell-cell aggregation

CHAPTER 6

Antifungal susceptibility to six antifungal agents

6.1 Introduction

Several antifungal agents, with different modes of action, are available for the treatment of oral *Candida* infections (Section 1.4, Table 1.4). The most common antifungal drugs in current clinical use are polyenes (amphotericin B and nystatin), azoles (fluconazole, miconazole, ketoconazole, itraconazole) and 5-fluorocytosine (5-FC). Recently, a new second-generation triazole (voriconazole) and an echinocandin antifungal agent (capsofungin) have been approved for clinical use by the Food and Drug Association (FDA) (Johnson & Kauffman, 2003), although these agents are only recommended for the treatment of systemic mycoses. Furthermore, other new triazoles (posaconazole, ravuconazole) are currently being developed for clinical use (Espinel-Ingroff, 1998; Espinel-Ingroff *et al*, 2001; Sanglard & Odds, 2002).

The increasing availability and clinical application of these antifungal agents has led to a rise in the frequency of reports of *in vitro* and *in vivo* antifungal resistance of *Candida* spp. (Pfaller *et al*, 1995; Espinel-Ingroff *et al*, 1999; Espinel-Ingroff *et al*, 2000). The emergence of resistance amongst pathogenic fungi is a function of the selective pressure of their exposure to antifungal drugs (White *et al*, 1998; Sanglard & Odds, 2002). Furthermore, antifungal resistance may depend on the frequency of exposure of susceptible fungal strains, the rate of growth of the fungus exposed to the drug, the number of mutations or phenotypic changes required for resistance, and time (Sanglard & Odds, 2002).

The failure (or success) of the treatment of candidal infection could be the result of primary (or intrinsic) and secondary (or acquired) fungal resistance but may also be

due to complex interactions occurring between the infecting fungus, the antifungal agent and the host environment. Primary (or intrinsic) resistance is that recorded before *in vivo* or *in vitro* antifungal drug exposure, while secondary (or acquired) resistance develops following exposure to an antifungal drug. Secondary resistance can be either reversible, due to transient adaptation, or irreversible, due to one or several genetic alterations (Sanglard & Odds, 2002; White *et al*, 2002).

Several yeast species (*C. glabrata, C. krusei, C. guilliermondii*, and *C. lusitaniae*) have a higher prevalence than others of primary resistance to amphotericin B, and *C. glabrata* and *C. krusei* are intrinsically less susceptible to triazoles than *C. albicans* itself. Furthermore, *C. dubliniensis* has been shown, *in vitro*, to rapidly develop stable resistance to fluconazole (Moran *et al*, 1998; White *et al*, 1998; Sanglard & Odds, 2002). In addition to these intrinsically resistant species, there are intrinsically resistant strains of *C. albicans* which may be part of the commensal microflora or may be acquired from the environment or other individuals (White *et al*, 1998).

The epidemiological changes in the susceptibility of pathogenic fungi to antifungal drugs have led to the standardisation of antifungal resistance assays *in vitro* and resistance breakpoint definitions. Firstly in 1992 and then in 1997 (National Committee for Clinical Laboratory Standards, 1997), the US National Committee for Clinical Laboratory Standards (NCCLS) proposed a reference method, M27-A, for antifungal susceptibility testing, specifying the breakpoints for several antifungal agents, such as 5-FC, itraconazole and fluconazole, thus permitting inter- and intra-laboratory reproducibility and comparison of antifungal susceptibilities for yeast pathogens. A second edition of the method, M27-A2, was released in 2002 (National Committee for Clinical Laboratory Standards, 2002). However, the NCCLS committee accepts that M27-A/M27-A2 have limitations (*i.e.* regarding the ability of the methods to distinguish between isolates' susceptible and resistant to amphotericin

B, to newer azoles and echinocandins and the time-consuming nature of the methods), and many clinical laboratories therefore prefer to use commercially available products that have previously shown correlation with M27-A (White *et al*, 1998; Sanglard & Odds, 2002). Several products based on variations of the NCCLS microdilution method are now available commercially. These include Candifast (International Microbio/Stago Group, Diagnostic International Distribution, Milan, Italy), Fungitest panel (Sanofi Diagnostic Pasteur, Paris, France now Bio-Rad), Integral System Yeasts (Liofilchem Diagnostic, L'Aquila, Italy), and Sensititre Yeast One (Trek Diagnostic Systems, Inc., Westlake, OH/Trek Diagnostic System Ltd., East Grinstead, England). Three recent studies compared one or more of these products with the NCCLS methodology (Posteraro *et al*, 2000; Chryssanthou, 2001; Morace *et al*, 2002). The Sensititre Yeast One product was studied in all evaluations, comparing favourably with the NCCLS M27-A methodology (Hospenthal *et al*, 2004). Both the Candifast and Integral System Yeast products compared poorly with the reference method. Fungitest was evaluated in the largest of these three studies (Morace *et al*, 2002). This multicenter comparative evaluation of six commercial systems and the NCCLS microdilution method M27-A was conducted on the fluconazole susceptibility of 800 *Candida* isolates (Morace *et al*, 2002). The results of the study indicated that Fungitest together with E-test (AB Biodisk, Slovna, Sweden) and Sensititre Yeast One can be considered useful for the *in vitro* evaluation of fluconazole susceptibility among *Candida* spp. isolates (Morace *et al*, 2002).

Although amphotericin B and 5-FC resistance has been reported, azole resistance appears to be emerging as the major problem in patients treated for yeast infections (Rex *et al*, 1995), possibly reflecting the relative use of these antifungals (White *et al*, 1998).

The supposed emergence of antifungal resistance, however, may partly reflect the use of non-standardised commercial systems for antifungal susceptibility testing and lack of agreement on the definition of a resistant fungal isolate. Indeed, large scale surveys on azole susceptibility of clinical yeast isolates based on the NCCLS method and relative MIC breakpoints do not support the belief that antifungal resistance represents a significant or increasing epidemiological problem (Sanglard & Odds, 2002).

It has been reported that poor glycaemic control associated with other local factors, such as the presence of oral dental prostheses, salivary pH, salivary flow rate and tobacco habits, could lead to the development of oral candidosis (Dorocka-Bobkowska *et al*, 1996). The use of topical antifungal agents to prevent the development of candidal infections in patients with poor metabolic control is frequent, particularly in elderly denture wearing patients. Elderly people frequently suffer from an unpleasant sensation in the mouth that may give rise to intolerable distress (Collin *et al*, 2000a). If the elderly are diabetic, typically type 2 DM, with poor glycaemic control, diabetologists and general practitioners tend to prescribe topical antifungal agents to prevent oral candidosis developing, even if signs of the disease are not present. The indiscriminate use of antifungal agents, even in DM populations, could determine changes in the prevalence of *Candida* spp. causing the disease and may then result in increased antifungal resistance.

The treatment options of superficial oral *Candida* infections (Sections 1.3 and 1.4), both in DM and non-DM subjects, are mainly based on the use of topical antifungal agents, such as azoles (mainly miconazole and fluconazole, but also ketoconazole and itraconazole) or polyenes (amphotericin B and nystatin) (Budtz-Jorgensen & Lombardi, 2000; Ellepola & Samaranayake, 2000b; Ellepola & Samaranayake, 2000a; Rex *et al*, 2000; Willis *et al*, 2001). In this study, we evaluated the antifungal

susceptibility of *Candida* isolates from DM patients to the most commonly used antifungal agents for the treatment of oral candidosis.

At the outset of this study, the use of the NCCLS microdilution reference method applied to different antifungal agents (those most commonly used as topical treatment) was considered. However, the adoption of a NCCLS method for all the antifungal agents we wished to analyse was ruled out because of lack of availability and the time-consuming nature of this method. Therefore, a commercially available methodology capable of rapidly assessing a range of antifungal susceptibilities of *Candida* isolates was used. This method, Fungitest, has proved useful in a multicenter comparative evaluation of six commercial systems (Morace *et al*, 2002) and was kindly provided by Prof. Polonelli's team at the University of Parma (Sezione di Microbiologia, Dipartimento di Patologia e Medicina di Laboratorio, Università di Parma, Italy).

Aim: The aim of this study was to assess the antifungal susceptibility of *Candida* isolates collected from patients affected by DM and from a group of healthy non-DM subjects to six commercially available antifungal agents.

Hypothesis: That there are differences in the antifungal susceptibility to six different antifungal agents of oral *Candida* isolates collected from patients with DM and from healthy non-DM subjects.

6.2 Methods

Oral *Candida* yeasts isolated from DM patients and from a control population (Section 4.3.1), were evaluated *in vitro* for their antifungal susceptibility to six

different antifungal agents using the commercial Fungitest kit, Bio-Rad, France (Section 2.8). Results were expressed by classification of the isolates as being 'resistant', 'intermediate resistant' or susceptible to each antifungal agent.

Fisher's exact test and Chi square test were used for the statistical analysis of categorical data, numeric data was analysed by the Student T test or ANOVA according to the postulates of each test, and differences within or between groups were considered significant when the probability (p) was less than or equal to 0.05.

Although there ~~are~~ were interesting variations in the number of isolates fully resistant to certain antifungal agents, it was not possible to undertake meaningful statistical analyses with all antifungal agents, as the number of resistant isolates in the other patient populations for the other antifungal agents was either too low or non-existent. Furthermore, the intention of this analysis was to determine the changes in antifungal sensitivity over a large number of geographically dispersed isolates from specific patient populations utilising a rapid screening method. The intention of this study was not to report accurate and clinically significant antifungal susceptibility on specific strains to a limited number of antifungal agents. Therefore, isolates with any enhanced resistance to the antifungal agents tested were grouped together for statistical analysis.

not sure about the sense of this sentence

6.3 Results

6.3.1 Antifungal susceptibility of *Candida* isolated from DM and non-DM subjects

6.3.1.1 Antifungal susceptibility of all *Candida* isolates

The resistance rates of all *C. albicans* isolates (177/229) tested were 9% (fluconazole), 17% (itraconazole and miconazole), 15% (ketoconazole), 7% (amphotericin B) and 5% (5-FC). Similarly, for non-*C. albicans* isolates (52/229), the

resistance rates for these antifungals were 29% (fluconazole and itraconazole), 35% (miconazole), 23% (ketoconazole), 6% (amphotericin B) and 13% (5-FC).

Interestingly, *Candida* isolates that expressed resistance or intermediate resistance to fluconazole were also found to exhibit resistance to at least one other antifungal agent. This finding of fluconazole cross-resistance with other antifungals was not encountered with any other combinations of antifungals (Appendix 2).

6.3.1.2 Association of *Candida* load (cfu/ml), species or genotype with antifungal resistance

There was no relationship evident between oral candidal load and the rates of antifungal resistance for the drugs tested ($p>0.05$) (Table 6.1a). It was, however, found that the non-*C. albicans* isolates exhibited higher resistant or intermediately resistant rates to fluconazole ($p=0.0008$) and miconazole ($p=0.01$) than *C. albicans* isolates (Table 6.1a). There was no equivalent relationship with susceptibility to ketoconazole, itraconazole, amphotericin B and 5-FC (Table 6.1a). All *C. albicans* group B isolates were susceptible to 5-FC.

6.3.1.3 Association of patient age, gender, tobacco habits and dental prostheses with antifungal resistance

There was no association between patient age or gender and the antifungal susceptibility of *Candida* ($p>0.05$). There were, however, fewer oral yeast isolated from patients without dentures that were resistant or intermediately resistant to miconazole ($p=0.02$) and 5-FC ($p=0.02$) (Table 6.1b). Furthermore, isolates from non-tobacco users had higher resistance or intermediate resistance to miconazole ($p=0.04$) or amphotericin B ($p=0.04$) (Table 6.1b).

6.3.1.4 Comparison of antifungal susceptibility between *Candida* from DM and non-DM subjects

Overall there were no significant differences in the antifungal susceptibility between isolates from DM and non-DM subjects (Table 6.2). In the case of DM isolates the antifungal resistance rates were 9% (itraconazole), 7% (ketoconazole), 4% (fluconazole) and 1% (5-FC). In comparison, resistance rates for non-DM isolates were 5% (itraconazole), 4% (ketoconazole), 1% (fluconazole) and 0% (5-FC). Similarly, intermediate resistance for DM recovered isolates was evident with fluconazole (10%), miconazole (21%), ketoconazole (13%), itraconazole (13%), amphotericin B (5%) and 5-FC (4%). In the case of non-DM recovered isolates the equivalent incidences of intermediate resistance were 11% (fluconazole), 21% (miconazole), 7% (ketoconazole) and 9% (itraconazole). Similarly, intermediate resistance was observed with rates of 11% for both amphotericin B and 5-FC. All remaining isolates from both DM and non-DM subjects were susceptible to the tested antifungals.

6.3.2 Antifungal susceptibility of isolates from DM patients

6.3.2.1 Effect of geographical location on antifungal resistance

Significant differences were observed in the antifungal susceptibility to the six different antifungal agents when the two DM populations from London and Parma were compared. Specifically, high resistance rates to itraconazole (12/83, 14%) and ketoconazole (11/83, 13%) were evident in *Candida* isolates from London DM patients. Interestingly, no resistance to miconazole, ketoconazole and amphotericin B were evident for Parma DM isolates (0/71, 0%).

Similarly, intermediate resistance rates of 13% for itraconazole (11/83) and 14% for ketoconazole (12/83) were encountered for London DM isolates. Equivalent intermediate resistance rates for Parma DM patients were 6% for fluconazole and

miconazole (4/71), 13% for itraconazole (9/71), 11% for ketoconazole (8/71), 1% for amphotericin B (1/71) and 3% for 5-FC (2/71).

Oral yeasts from London DM patients also had higher resistance (or intermediate resistance) to fluconazole ($p=0.02$), miconazole ($p<0.0001$), and ketoconazole ($p=0.01$) than the isolates from Parma DM patients. There was also a trend of increased resistance of isolates from London DM patients to itraconazole and 5-FC ($p=0.08$) (Table 6.3).

6.3.2.2 Effect of specific DM properties on antifungal resistance

When antifungal susceptibility was compared against type of DM, DM duration and haemoglobin glycosylation levels, no statistically relevant associations could be drawn. However, it was apparent that *Candida* isolated from DM patients with neuropathy and retinopathy were more susceptible to miconazole ($p=0.04$) than those strains from patients who had nephropathy (Table 6.4).

No statistically significant ($p>0.05$) association was found between antifungal susceptibility and patient age, gender, tobacco usage or denture wearing among the two DM populations.

No significant associations between resistance to any tested antifungal agent and the level of oral yeast colonisation (cfu/ml), or *C. albicans* genotype were found among DM patients from London and from Parma (Table 6.5).

6.4 Discussion

The present study examined the antifungal susceptibility of oral yeast isolated from patients with and without DM. The objective of the study was to establish whether increased antifungal resistance was evident for DM isolates, which may lead to problems in the management of patients prone to oral candidal infections. No

differences in the antifungal susceptibility to the six agents tested were observed between *Candida* isolates from DM and non-DM subjects. However, differences were observed between the two geographically different DM populations.

The higher incidence of intermediate resistance in London DM isolates to certain antifungals was probably not related to recent exposure to antifungal treatment, since none of these patients had received antifungal drugs in the preceding six months. However, their exposure to antifungal agents prior to this time was unknown. Therefore, it may well be that significant variation in lifetime antifungal exposure existed between the two DM populations and that this lifetime antifungal exposure variation is the reason for the elevated antifungal resistance rates observed in the London DM population. Interestingly, one of the most common antifungal agents used to treat patients with dentures suffering from oral candidosis is miconazole (Dias *et al*, 1997). It could be presumed that, although patients in the present study had not taken any antifungal agents in the preceding 6 months, those patients with dentures possibly had increased exposure to this antifungal agent during their lifetime. This may well account for the increased level of resistance to miconazole observed in those patients who wore dentures in the present study, and indicates that lifetime antifungal exposure may well be more significant than antifungal exposure in the preceding 6 month period.

The difference in the antifungal resistance of isolates from the two populations of DM patients may relate to differences in the therapeutic management of candidal infections between the two centres in Italy and the UK, since the introduction of these antifungal agents in the two countries. However, there was no available long-term data to support this notion. Although no association could be observed in the diabetic status of the patients in London and Parma, it could be assumed that as the London group had more long-standing DM with more complications (Chapter 3, Section

3.3.2), they would be more likely to have received or taken the antifungal agents themselves.

As has been reported in literature (Rex *et al*, 2000; Sanglard & Odds, 2002), it is more likely that intermittent antifungal therapy leads to the development of azole resistance rather than long-term administration, as may be the case with the present group of DM patients in London.

The *Candida* isolated from the oral cavities of the DM patients resident in London, demonstrating *in vitro* resistance to the different antifungal agents tested, may have acquired resistance as a consequence of the selective pressure of azole treatment (Sanglard & Odds, 2002).

The most important clinical consequence of antifungal resistance usually manifests itself as a failure to successfully treat patients affected by candidosis and in changes in the prevalence of *Candida* spp. causing the disease (Sanglard & Odds, 2002).

Interestingly, none of the patients with resistant *Candida* species had signs and symptoms of oral *Candida* infections, reflecting that a patient's own immunity system can control the yeast pathogenicity. This was observed for nearly all the patients studied in the thesis.

Although some of the yeasts isolated from the DM population from London (6/83, 7.2%) and from the control group (8/75, 10.6%) showed an *in vitro* intermediate resistance to amphotericin B, as noted above, none of the *Candida* isolates from the three groups of patients enrolled in the study were completely resistant to amphotericin B. This antifungal drug (Epstein & Polsky, 1998; Lefebvre & Domenge, 2002) is the most widely used in the UK as a topical agent (lozenges, suspensions) for the treatment of oral candidosis, and as these topical formulations are not available in Italy, it is possible that this geographical variation might explain the trends for increased amphotericin resistance in UK isolates. In any case,

secondary resistance to amphotericin B generally seems to be an infrequent development (White *et al*, 1998). However, the methodologies and results of the present study do not clarify whether the antifungal resistance of examined isolates is intrinsic or acquired. In addition, because DM has had little effect on the presence of antifungal resistance of oral yeast isolates, there remains the possibility that any resistance shown has been acquired from family members (or indeed other hospital patients) (Sanglard & Bille, 2002; Sanglard & Odds, 2002; Sullivan & Coleman, 2002).

It is well known that wearing acrylic dentures is an important predisposing factor for the development of oral candidosis, as these prostheses, when ill-fitting and not kept clean, act as a reservoir for infection (Ellepola *et al*, 1990; Ellepola & Samaranayake, 2000a). Furthermore, increased oral candidal load is much more common in full-denture wearers than in dentate individuals (Parvinen *et al*, 1994; Manfredi *et al*, 2002). *Candida*-induced denture stomatitis is most commonly treated with the polyene antifungal agent nystatin and, where available, with an amphotericin B suspension. Both drugs are used topically and applied to the fitting surface of the denture before insertion (Cawson, 1965). Topical miconazole (oral gel, lacquer) is also a viable treatment option for patients affected by this form of oral candidosis (Konsberg & Axell, 1994; Parvinen *et al*, 1994; Dias *et al*, 1997).

Despite the availability of a range of antifungal agents for the treatment of oral *Candida* infections, therapy frequently fails. In the mouth, the diluent effect of saliva and the cleansing action of the oral musculature may also reduce the levels of antifungals to below their effective therapeutic concentrations (Ellepola *et al*, 1990; Dorocka-Bobkowska *et al*, 2003). The formation of *Candida* biofilms further reduces the susceptibility of yeasts to antifungal agents (Douglas, 2002; Douglas, 2003).

In this study, isolates from denture wearers were less susceptible *in vitro* to miconazole and 5-FC than isolates collected from dentate patients. It can be hypothesised that the *in vitro* resistance of these *Candida* isolates is a consequence of the past widespread topical use of miconazole for the treatment of *Candida*-induced denture stomatitis.

5-fluorocytosine is a DNA analogue, mainly used for the management of systemic *Candida* infections, particularly in immunocompromised patients, but not for the treatment of oral candidosis. However, it is also well known that *Candida* spp., and *C. albicans* in particular, rapidly become resistant *in vitro* to this agent and therefore, when treating severe deep systemic candidosis, 5-FC is usually combined with another antifungal agent, such as amphotericin B (Ellepola & Samaranayake, 2000a).

The analysis of the susceptibility of all the oral *Candida* isolates (collected from the three groups of patients enrolled in the study) to the different antifungal agents has shown that *C. albicans* strains were more susceptible to fluconazole and miconazole than non-*C. albicans* strains.

It has been demonstrated that *C. albicans* is usually susceptible to all major antifungal agents (Rex *et al*, 2000), and other *Candida* spp., such as *C. glabrata* and *C. krusei*, are intrinsically less susceptible to triazole and amphotericin B (Sanglard & Odds, 2002). However, *C. albicans* resistance to different antifungal agents, such as triazoles, and fluconazole particularly, has recently been reported among different groups of immunocompromised patients, where this agent is frequently used in prophylaxis and treatment of fungal infections (Rex *et al*, 2000; Rex *et al*, 2001; Vazquez *et al*, 2001).

Furthermore, although the susceptibility of *Candida* spp. to the available antifungal agents can be predicted if the species of the infecting isolate is known, individual

isolates do not necessarily follow the general pattern (Pfaller *et al*, 1994; Pfaller *et al*, 1998c; Rex *et al*, 2000).

Several studies have reported that multiple genes are involved in the process of resistance to azoles. For example, CaMDR1, one of the MF transporter genes upregulated in some *C. albicans*-resistant strains, expressed selectivity among the azoles, resulting in cells becoming resistant to fluconazole but not to ketoconazole or itraconazole (Alarco *et al*, 1997; Balan *et al*, 1997; Balkis *et al*, 2002). Interestingly though, in the present study, isolates that were resistant or showed intermediate resistance to fluconazole were also resistant to at least one other antifungal agent. This was not the case for any of the other antifungal agents tested (Appendix 2).

For this reason, susceptibility testing for azole resistance is increasingly important in the management of candidosis.

The findings in this study confirm the results of others who indicated that the previously reported emergence of *C. albicans* fluconazole and, more generally, triazole resistance, was probably mainly due to the non-standardised susceptibility methods rather than a real increase in the resistance of *C. albicans* species (Sanglard & Odds, 2002). However, the issue is still controversial. The commercial method (Fungitest, Bio-Rad) used in the present study and is considered a useful kit for the *in vitro* evaluation of fluconazole sensitivity among *Candida* spp. isolates in clinical laboratories, with a positive agreement among laboratories regarding its reproducibility. Although this method provides a limited number of drug concentrations and as a commercial kit, has some limitations compared to the NCCLS reference methods, it is regarded as one of the easiest and most rapid commercial kits available (Morace *et al*, 2002). Furthermore, the NCCLS M27-A antifungal concentration breakpoints are only available for three of the most common drugs used (fluconazole $\leq$ 8 - $\geq$64 μg/ml, itraconazole $\leq$0.125 - $\geq$1 μg/ml and 5-FC $\leq$4 - $\geq$32

μg/ml) (National Committee for Clinical Laboratory Standards, 1997). Fungitest breakpoints are specified by the manufacturer (Bio-Rad) and have been selected following the study of the distribution of the antifungal agents' Minimal Inhibitory Concentrations (MIC) obtained with prototype microplates used with the same procedure as Fungitest. Moreover, breakpoints of Fungitest for fluconazole are exactly the same as those in the NCCLS reference method.

The results obtained in this study seem to confirm that the rates of triazole resistance in *Candida* spp. are low and possibly overestimated (Sanglard & Odds, 2002) perhaps suggesting that the associated clinical problem could be less serious than hypothesised. However, because the present non-*C. albicans* spp. isolates were less susceptible to fluconazole and miconazole (Table 6.6b) than *C. albicans* isolates, the increased use of "over the counter" azole formulations could result in increased oral carriage of non-*C. albicans* and thus the possibility of acquisition by, and infection of, immunocompromised groups (White *et al*, 1998; Sanglard & Odds, 2002).

Finally, it has been reported (Mercure *et al*, 1993) that *C. albicans* genotype B, which harbours a 379 nucleotide (Group I intron) in the 25S rDNA and *C. albicans* genotype C, a mixture of intron-containing and 'intron-less' 25S rDNA, both showed a greater susceptibility to 5-FC than the intronless strain *C. albicans* genotype A. This observation was further confirmed by other studies (McCullough *et al*, 1999b). The different levels of susceptibility to this agent by these subgroups of *C. albicans* seemed to be due to the inhibitory effect of biosynthetic incorporation of this base analogue into the group I intron ribozymes (Mercure *et al*, 1993).

However, in this study no statistical differences were observed in the susceptibility to 5-FC of the *C. albicans* subgroup B isolates from all the patients evaluated in the study, and only one isolate from the *C. albicans* subgroup C (Appendix 2, C/M 29) expressed intermediate resistance to the same agent. This finding is in agreement with

others (Tamura *et al*, 2001) and suggests that the contribution of the group I intron to the susceptibility of *Candida* spp. to 5-FC may only be one factor, and that other factors (von Ahsen *et al*, 1991; von Ahsen & Schroeder, 1991) may play more important roles in 5-FC susceptibility of *Candida* spp.

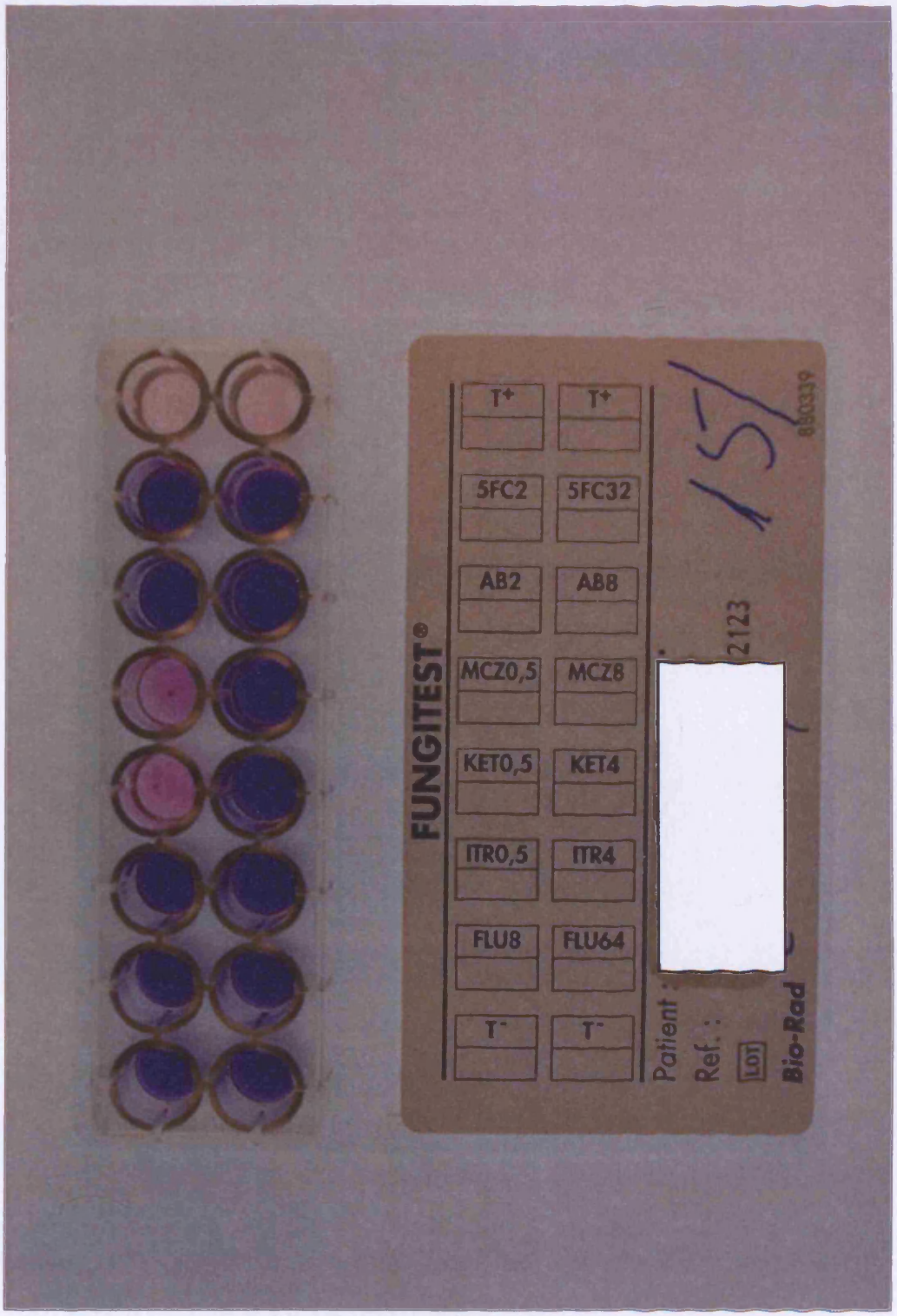

Figure 6.1: Example of Fungitest (Bio-Rad) commercial kit

This *C. albicans* isolate expressed intermediate resistance to miconazole and ketoconazole (pink-blue). T+: positive control test; 5 FC2: 5-FC at 2 µg/ml; 5FC32: 5-FC at 32 µg/ml; AB 2: amphotericin B at 2 µg/ml; AB 8: amphotericin B at 8 µg/ml; MCZ 0.5: miconazole at 0.5 µg/ml; MCZ 8: miconazole at 8 µg/ml; KET 0.5: ketoconazole at 0.5 µg/ml; KET 4: ketoconazole at 4 µg/ml; ITR 0.5: itraconazole at 0.5 µg/ml; ITR 4: itraconazole at 4 µg/ml; FLU 8: fluconazole at 8 µg/ml; FLU 64: fluconazole at 64 µg/ml; T-: negative control test

Table 6.1 a: Analysis of the susceptibility of the *Candida* strains isolated from the oral cavity of all the *Candida* carriers to six different antifungal agents

Total patients	F S	F IR	p value	M S	M IR	p value	KS	K IR	p value	I S	I IR	p value	A S	A IR	p value	5FC S	5FC IR	p value
<100 cfu	105	14	p=0.4447	98	21	p=0.1967	99	20	p=1.0000	95	24	p=0.8390	113	6	p=0.4260	113	6	p=0.3014
>100 cfu	93	17		82	28		91	19		89	21		101	9		100	10	
C. albicans	**161**	**16**	**p=0.0008**	**147**	**30**	**p=0.0108**	150	27	p=0.2093	147	30	p=0.0734	165	12	p=1.0000	168	9	p=0.0580
Non-*C. albicans*	**37**	**15**		**34**	**18**		40	12		37	15		49	3		45	7	
***C. albicans* A**	130	15	p=0.4131	121	24	p=0.7033	122	23	p=0.5189	119	26	p=0.7522	135	10	p=0.7516	137	8	p=0.2694
***C. albicans* B**	24	1		21	4		21	4		22	3		23	2		25	0	
***C. albicans* C**	7	0		5	2		7	0		6	1		7	0		6	1	

Data was subdivided according to patient *Candida* colonisation (cfu/ml), species and *C. albicans* genotype subgroups
Statistical analysis was performed using Fisher's exact and Chi square parametric tests
FS: fluconazole susceptible; F IR: fluconazole intermediately resistant/resistant; MS: miconazole susceptible; M IR: miconazole intermediately resistant/resistant; KS: ketoconazole susceptible; K IR: ketoconazole intermediately resistant/resistant; I S: itraconazole susceptible; I IR: itraconazole intermediately resistant/resistant; A S: amphotericin B susceptible; A IR: amphotericin B intermediately resistant/resistant; 5-FC S: 5-FC susceptible; 5FC IR: 5-FC intermediately resistant/resistant

Table 6.1b: Analysis of the susceptibility of the *Candida* strains isolated from the oral cavity of all the *Candida* carriers to six different antifungal agents

Total patients	F S	F IR	p value	M S	M IR	p value	KS	K IR	p value	I S	I IR	p value	A S	A IR	p value	5-FC S	5-FC IR	p value
Male	99	17	p=0.7000	95	21	p=0.2600	91	25	p=0.1692	89	27	p=0.1851	109	7	p=0.7948	108	8	p=1.0000
Female	99	14		85	28		97	16		95	18		105	8		105	8	
<60 years old*	99	12	p=0.4206	89	22	p=0.6216	93	18	p=1.0000	92	19	p=0.4927	106	5	p=0.7650	108	3	p=0.0789
>60 years old*	91	16		82	25		89	18		84	23		101	6		98	9	
Dentate	108	14	p=0.3411	**103**	**19**	**p=0.0244**	98	24	p=0.2930	99	23	p=0.8678	113	9	p=0.7900	**118**	**4**	**p=0.0211**
Dentures	90	17		**77**	**30**		92	15		85	22		101	6		**95**	**12**	
Tobacco users	43	5	p=0.6363	**43**	**5**	**p=0.0466**	41	7	p=0.8290	38	10	p=0.867	**48**	**0**	**p=0.0451**	45	3	p=1.0000
Non-tobacco users	155	26		**137**	**44**		149	32		146	35		**166**	**15**		168	13	

Data was subdivided according to patient gender, age, denture status and smoking habits
Statistical analysis was performed using Fisher's exact and Chi square parametric tests
FS: fluconazole susceptible; F IR: fluconazole intermediately resistant/resistant; MS: miconazole susceptible; M IR: miconazole intermediately resistant/resistant; KS: ketoconazole susceptible; K IR: ketoconazole intermediately resistant/resistant; I S: itraconazole susceptible; I IR: itraconazole intermediately resistant/resistant; A S: amphotericin B susceptible; A IR: amphotericin B intermediately resistant/resistant; 5-FC S: 5-FC susceptible; 5-FC IR: 5-FC intermediately resistant/resistant
* Of the twelve non-DM control subjects who did not give their date of birth (special needs patients), 10 harboured oral yeasts. Of these ten, 9 yielded one *Candida* strain each and one additional non-DM subject yielded two *Candida* strains

Table 6.2: *In vitro* susceptibility to six different antifungal agents of oral *Candida* isolates from patients with and without DM

Antifungal agents	DM patients	Non-DM subjects	p value
Fluconazole			
S	132/154 (86%)	66/75 (88%)	p = 0.57
I	16/154 (10%)	8/75 (11%)	
R	6/154 (4%)	1/75 (1%)	
Miconazole			
S	122/154 (79%)	59/75 (79%)	
I	32/154 (21%)	16/75 (21%)	
R	0/154	0/75	p = 1.00
Ketoconazole			
S	123/154 (80%)	67/75 (89%)	
I	20/154 (13%)	5/75 (7%)	
R	11/154 (7%)	3/75 (4%)	p = 0.20
Itraconazole			
S	120/154 (78%)	64/75 (85%)	
I	20/154 (13%)	7/75 (9%)	
R	14/154 (9%)	4/75 (5%)	p = 0.40
Amphotericin B			
S	147/154 (95%)	67/75 (89%)	
I	7/154 (5%)	8/75 (11%)	
R	0/154	0/75	p = 0.09
5-FC			
S	146/154 (95%)	67/75 (89%)	
I	6/154 (4%)	8/75 (11%)	
R	2/154 (1%)	0/154	p = 0.08

Statistical analysis was performed using Fisher's exact and Chi square parametric tests
S: susceptible; I: intermediately resistant; R: resistant

Table 6.3: Analysis of the antifungal susceptibility of the *Candida* species isolated from the oral cavities of London and Parma patients with DM

Antifungal agents	DM London patients	DM Parma patients	p value
Fluconazole			
S	66/83 (79.5%)	66/71 (93%)	
IR	17/83 (20.5%)	5/71 (7%)	**p = 0.02**
Miconazole			
S	55/83 (66.3%)	67/71 (94.4%)	
IR	28/83 (33.7%)	4/71 (5.6%)	**p <0.0001**
Ketoconazole			
S	60/83 (72.3%)	63/71 (88.7%)	
IR	23/83 (27.7%)	8/71 (11.3%)	**p = 0.01**
Itraconazole			
S	60/83 (72.3%)	60/71 (84.5%)	
IR	23/83 (27.7%)	11/71 (15.5%)	p = 0.08
Amphotericin B			
S	77/83 (92.8%)	70/71 (98.6%)	
IR	6/83 (7.2%)	1/71 (1.4%)	p = 0.08
5-FC			
S	78/83 (94%)	68/71 (95.8%)	
IR	5/83 (6%)	3/71 (4.2%)	p = 0.72

Statistical analysis was performed using Fisher's exact and Chi square parametric tests
S: susceptible; IR: intermediately resistant/resistant

Table 6.4: Analysis of the susceptibility of the *Candida* strains isolated from the oral cavity of patients with DM to six different antifungal agents

DM patients	FS	F IR	p value	M S	M IR	p value	K S	K IR	p value	I S	I IR	p value	A S	A IR	p value	5-FC S	5-FC IR	p value
Male	67	12	p=0.8199	64	15	p=0.5562	59	20	p=0.1115	57	22	p=0.0838	75	4	p=1.0000	75	4	p=1.0000
Female	65	10		57	18		64	11		63	12		72	3		71	4	
<60 years old	58	9	p=0.8211	53	14	p=1.0000	52	14	p=0.8401	52	14	p=0.8472	63	3	p=1.0000	65	1	p=0.1388
>60 years old	74	13		68	19		71	17		68	20		84	4		81	7	
Dentate	67	10	p=0.8184	62	14	p=0.4341	57	19	p=0.1618	59	17	p=1.0000	73	3	p=1.0000	75	1	p=0.0634
Dentures	65	12		59	19		66	12		61	17		74	4		71	7	
Tobacco users	27	3	p=0.5711	27	3	p=0.1348	25	5	p=0.8003	23	7	p=0.8109	30	0	p=0.3466	30	0	p=0.3559
Non-Tobacco users	105	19		94	30		98	26		97	27		117	7		116	8	
DM type 1	37	5	p=0.7969	34	8	p=0.8260	31	11	p=0.2651	33	9	p=1.0000	42	0	p=0.1906	41	1	p=0.4480
DM type 2	95	17		87	25		92	20		87	25		105	7		105	7	
<10 years DM*	48	8	p=1.0000	45	11	p=1.0000	46	10	p=0.6632	41	15	p=0.4025	53	3	p=0.6982	52	4	p=0.7219
>10 years DM*	65	11		60	16		59	17		61	15		73	3		72	4	
Neuropathy	24	7	p=0.1633	**23**	**8**	**p=0.0475**	23	8	p=0.7799	24	7	p=0.8531	28	3	p=0.2969	28	3	p=0.9846
Nephropathy	16	7		**17**	**9**		16	4		16	4		18	2		18	2	
Retinopathy	38	5		**36**	**4**		33	13		34	12		45	1		41	5	
$HbA1_c$ <7.5%	38	9	p=0.5203	32	15	p=0.0720	35	12	p=0.4424	34	13	p=0.4882	44	3	p=0.6958	43	4	p=0.4194
$HbA1_c$ >7.5%; <8.5%	36	5		36	5		35	6		32	9		39	2		40	1	
$HbA1_c$ >8.5%	58	8		53	13		53	13		54	12		64	2		63	3	

Data was subdivided according to patient gender, age, denture status, type and duration of DM, long-term diabetic complications and glycaemic control ($HbA1_c$). Statistical analysis was performed using Fisher's exact and Chi square parametric tests

* For 36 DM patients from London it was not possible to establish the duration of their disease. Of these, 22 harboured oral yeasts

FS: fluconazole susceptible; F IR: fluconazole intermediately resistant/resistant; MS: miconazole susceptible; M IR: miconazole intermediately resistant/resistant; KS: ketoconazole susceptible; K IR: ketoconazole intermediately resistant/resistant; I S: itraconazole susceptible; I IR: itraconazole intermediately resistant/resistant; A S: amphotericin B susceptible; A IR: amphotericin B intermediately resistant/resistant; 5-FC S: 5-FC susceptible; 5-FC IR: 5-FC intermediately resistant.

Table 6.5: Analysis of the susceptibility of the *Candida* strains isolated from the oral cavity of the two groups of patients with DM to six different antifungal agents

DM patients	FS	F IR	p value	M S	M IR	p value	K S	K IR	p value	I S	I IR	p value	A S	A IR	p value	5-FC S	5-FC IR	p value
<100 cfu	84	11	p=0.2432	78	17	p=0.2255	77	18	p=0.6822	75	20	p=0.6948	91	4	p=1.0000	89	6	p=0.7112
>100 cfu	48	11		43	16		46	13		45	14		56	3		57	2	
C. albicans	**109**	**12**	**p=0.0088**	**101**	**20**	**p=0.0270**	98	23	p=0.6243	97	24	p=0.2370	116	5	p=0.6427	116	5	p=0.3694
Non-*C. albicans*	**23**	**10**		**21**	**12**		25	8		23	10		31	2		30	3	
***C. albicans* A**	88	11	p=0.6010	83	16	p=0.3189	79	20	p=0.5258	78	21	p=0.6655	95	4	p=0.8398	94	5	p=0.5602
***C. albicans* B**	16	1		15	2		14	3		15	2		16	1		17	0	
***C. albicans* C**	5	0		3	2		5	0		4	1		5	0		5	0	

Data was subdivided according to patient *Candida* colonisation (cfu/ml), species and *C. albicans* genotype subgroups
Statistical analysis was performed using Fisher's exact and Chi square parametric tests
FS: fluconazole susceptible; F IR: fluconazole intermediately resistant/resistant; MS: miconazole susceptible; M IR: miconazole intermediately resistant/resistant; KS: ketoconazole susceptible; K IR: ketoconazole intermediately resistant/resistant; I S: itraconazole susceptible; I IR: itraconazole intermediately resistant/resistant; A S: amphotericin B susceptible; A IR: amphotericin B intermediately resistant/resistant; 5-FC S: 5-FC susceptible; 5-FC IR: 5-FC intermediately resistant/resistant

CHAPTER 7

Analysis of the strain relatedness of *C. albicans* isolates utilising multiple PCR fingerprints

7.1 Introduction

The increasing incidence of oral candidosis, particularly in immunocompromised patients, and the emergence of strains resistant to conventional antifungal therapies, has led to the implementation of laboratory techniques that allow the rapid identification of causative organisms and the study of *Candida* strains at species levels.

To increase our understanding of *Candida* pathogenicity, the identification of those strains most frequently associated with infections is of paramount importance.

Candida spp., and *C. albicans* in particular, are ubiquitous organisms that can be acquired endogenously from the host's normal flora or exogenously from the environment, from infected patients or from health care workers. The ability to identify particularly infective strains allows outbreaks of infections to be monitored and cross-infection controls to be assessed (Sullivan & Coleman, 2002).

In addition, strain identification can establish whether relapses of infection are due to a novel infecting organism or to persistence/re-infection by the original strain. Furthermore, it is important to remember that more than one strain can be involved in an infection (Soll, 2000; Sullivan & Coleman, 2002).

Specific techniques can also determine the dynamics of yeast populations and allow the study of association between virulence or drug resistance and specific clones or clusters of related strains.

Traditionally, the methods used to characterise and speciate *Candida* strains were based on the analysis of phenotypic traits, such as colony morphology (and colour indicator media), biochemical and serological tests, characteristic carbohydrate assimilation profiles and chemical resistance patterns (Chapter 1, Section 1.6). These methods are easy to perform, but they are not particularly specific since unrelated strains of *Candida*, and sometimes even different species, often share phenotypic characteristics (Sullivan & Coleman, 2002).

For this reason, strain differentiation methods have been developed based on the analysis of genotypic differences. Genotypic methods based on nucleic acid analysis offer greater differentiation and reproducibility for strain delineation than phenotyping (McCullough *et al*, 1996; Bartie *et al*, 2001).

PCR-based strategies, such as PCR fingerprinting and Randomly Amplified Polymorphic DNA (RAPD) methods (Sections 1.6.3.1-1.6.3.2; Table 1.7) have been used in particular to investigate cluster infections caused by identical or similar *Candida* strains (Shin *et al*, 2000), the emergence of resistant strains during antifungal therapy (Metzgar *et al*, 1998), the colonisation pattern of strains in different clinical situations and the microevolution of strains within a particular species (Lockhart *et al*, 1997; Pujol *et al*, 1997; Enger *et al*, 2001; Dassanayake & Samaranayake, 2003a). These methods are extremely effective in differentiating and determining reproducibility; they require minimum starting material and are rapid and simple to perform (Lischewski *et al*, 1995; Barchiesi *et al*, 1997; Carlotti *et al*, 1997; Clemons *et al*, 1997; Diaz-Guerra *et al*, 1997; Meyer *et al*, 1997).

Different PCR fingerprinting studies have been used to investigate *C. albicans* types associated with particular disease conditions, anatomical sites or geographical areas (Soll, 2000; Bartie *et al*, 2001). Particularly, it has recently been shown that certain related groups of *C. albicans* clones may be endemic in specific hospitals and

geographic areas (Clemons *et al*, 1997; Pfaller *et al*, 1998b; McCullough *et al*, 1999b; Schmid *et al*, 1999). Furthermore, a discrete genetic cluster of fluconazole-resistant *C. albicans* strains recovered from separate HIV-infected patients has recently been observed (Xu *et al*, 2000; Sullivan & Coleman, 2002). This finding supports the hypothesis that resistance occurred independently or that a resistant strain was spread by horizontal transmission among patients and highlights the potential value of strain characterisation in elucidating pathogenic and epidemiological traits.

In this study, *C. albicans* genetic relatedness was assessed for two geographically different patient groups (London, UK and Parma, Italy) affected by DM. In this way, it would be possible to establish a geographic variation amongst oral *C. albicans* strains that may be indicative of local diabetic treatment, including antifungal use. Furthermore, *C. albicans* samples from oral cavities of non-DM healthy subjects were also analysed by PCR fingerprinting to evaluate the possible genetic differences among diabetic endogenous strains and non-DM ones. To the best of my knowledge, this is the first study to evaluate the genetic variability of the *C. albicans* strains among DM patients and non-DM subjects using PCR fingerprinting methods.

Aim: The aim of this study was to assess the genetic variability of the oral *C. albicans* strain among DM patients from different localities, and amongst DM patients and a group of non-DM subjects.

Hypothesis: That there are genetic differences between *Candida* isolates from DM patients and non-DM subjects as a result of differing and distinct oral environments. Furthermore, it is hypothesised that London and Parma DM patients will carry genetically distinct *Candida* strains arising from the respective different treatment regimens.

7.2 Material and methods

Candida albicans isolates were collected as previously described (Sections 2.2). All isolates were well characterised and had been defined at species level as *C. albicans* (Sections 2.4-2.5). The analysis attempted to evaluate the genetic differences of *C. albicans* isolates from the 3 different groups of patients evaluated in this study. The analysis did not include other *Candida* spp., which may present a greater genetic variability. The sample size of 30 *C. albicans* isolates within each group was based on similar earlier comparative trials (Xu *et al*, 2000) and analyses showed that this number was needed to achieve 80% power for revealing genetic differences, if present.

Genomic DNA was extracted from all isolates (Section 2.4.2). Five separate PCR fingerprinting techniques were performed to genotypically assess strain similarity. Each of these techniques used a specific single primer (Table 7.1) and controlled thermocycler parameters as outlined in Section 2.5.2. All PCR amplicons were separated in 2% agarose gel in TAE buffer for 2 h at 2 V/cm, and visualised by ultraviolet transillumination following ethidium-bromide staining.

The results of the 5 PCR fingerprinting methods were combined by selecting 3 to 5 polymorphic bands for each method, resulting in 17 polymorphic bands that were shown to be widely spread through the genome. Each band was scored as either present or absent. These results were phylogenetically analysed using the computer program PAUP 4.08. Simple analyses were carried out using the optimal criterion as distance and pair-wise distances between each of the isolates measured. To aid visualization of the relationship between isolates based on this analysis, a phenogram was generated by the unweighted pair-group method with arithmetic mean (UPGMA) (Nei, 1987). Ties (if encountered) were broken systematically and the distance

measure was set to mean character difference. The statistical analysis of the present chapter was performed by Dr M. J. McCullough.

7.3 Results

7.3.1 Comparison of genetic variability between DM patients and non-DM subjects

Two *C. albicans* isolates (1 from UK non-DM control patients and 1 from Italian DM patients) previously identified and studied did not re-grow. Twenty-nine *C. albicans* isolates from the non-DM control UK patients, 30 from the DM UK patients and 30 from the DM Italian patients were selected (total of 89 *C. albicans* isolates) at random and analysed using the results from all 5 fingerprinting methods. Each isolate was given a designation consisting of 17 digits. All 17 of the polymorphic bands chosen were parsimony-informative. These digits were either 0 or 1, depending on whether the polymorphic band was either absent or present respectively. For example, Table 7.2 shows the genotypic designation of 8 randomly chosen *C. albicans* isolates. The number of differences in the bands between strains was measured.

Figures 7.1-7.5 show examples of electrophoretic separation of PCR fingerprinting using the 5 different primers.

Statistical analyses of these PCR fingerprint banding pattern differences (Table 7.3) showed that there was an average of 3.24 differences between all isolates and genotype B; the C isolate group was significantly more genetically coherent (mean difference of 2.23 bands) than the genotype A *C. albicans* isolate group (mean difference of 3.72 bands, $p < 0.0001$). Moreover, the isolates from the Italian DM patients were the most genetically cohesive group, with a mean difference of only 2.93 bands; the isolates from the control patients had 3.09 mean differences and the isolates from the UK DM patients had significantly more genetic diversity with a mean number of differences between isolates of 3.65 ($p < 0.0001$; Table 7.3).

Phylogenetic analysis of these isolates was graphically depicted by a phylogram, shown in Figure 7.6. The distance shown at the bottom of this phylogram of 0.1 is the distance within which strains are 90% similar. The 89 *C. albicans* isolates studied can thus be grouped and sub-grouped based on this phylogenetic analysis. Table 7.4 shows that these isolates can be divided into 4 large genotypic groups (A to D) that are co-located on the phylogram (Figure 7.6) and are more than 90% genotypically similar, while the other 17 genotypic sub-groups (A1-A17) are co-located and more than 95% similar (Table 7.4)

Table 7.1: Designation and sequence of the 5 primers used in each of the 5 distinct PCR fingerprinting techniques for phlyogenetic analysis

Designation	Sequence 5' – 3'
TELO	TGG GTG TGT GGG TGT GTG GGT GTG
GACA	GAC AGA CAG ACA GAC A
M13	GAC GGT GGC GGT TCT
OPA-03	AGT CAG CCA C
T3B	AGG TCG CGG GTT CGA ATC

Table 7.2: Genetic strain designation of 8 randomly selected isolates based on the 17 polymorphic PCR fingerprinting methods

Strain designation	TELO	GACA	M13	OPA-03	T3B
A	0100	010	011	11	10100
B	0100	110	011	11	11101
C	0010	110	011	10	00001
D	0010	110	011	01	00011
E	0101	110	011	11	11101
F	0100	110	011	11	11101
G	0100	010	011	11	11101
H	0101	110	111	11	11101

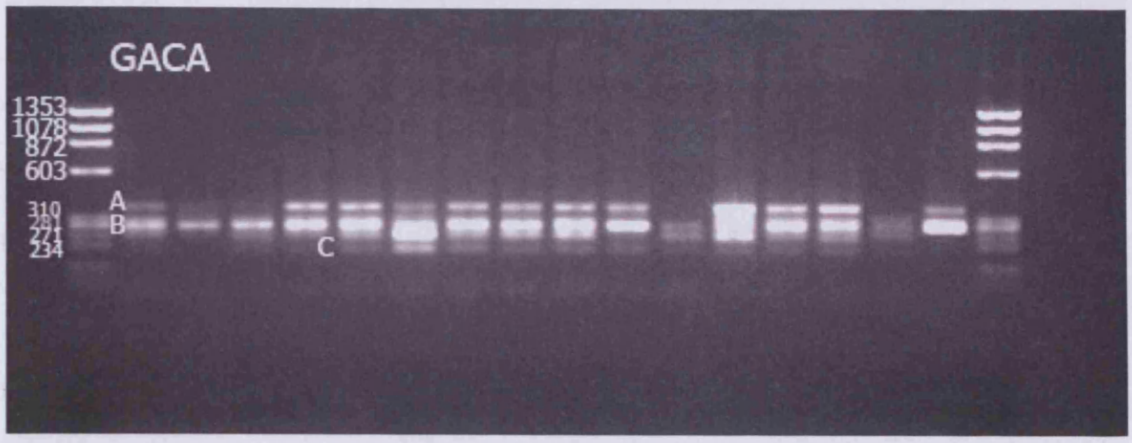

Figure 7.1: Examples of electrophoretic separation of PCR fingerprinting using (GACA)4 sequence

Three bands were selected for evaluation in this study: A: ~310bp, B: ~281 bp; C:~ 234 bp

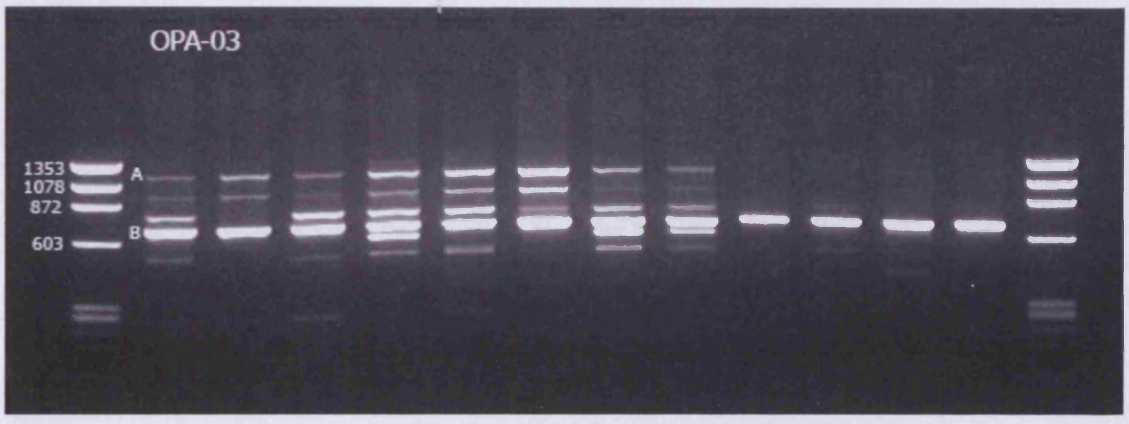

Figure 7.2: Examples of electrophoretic separation of PCR fingerprinting using OPA-03 sequence

Two bands were selected for evaluation in this study: A: ~1053 bp; B: above 603 bp

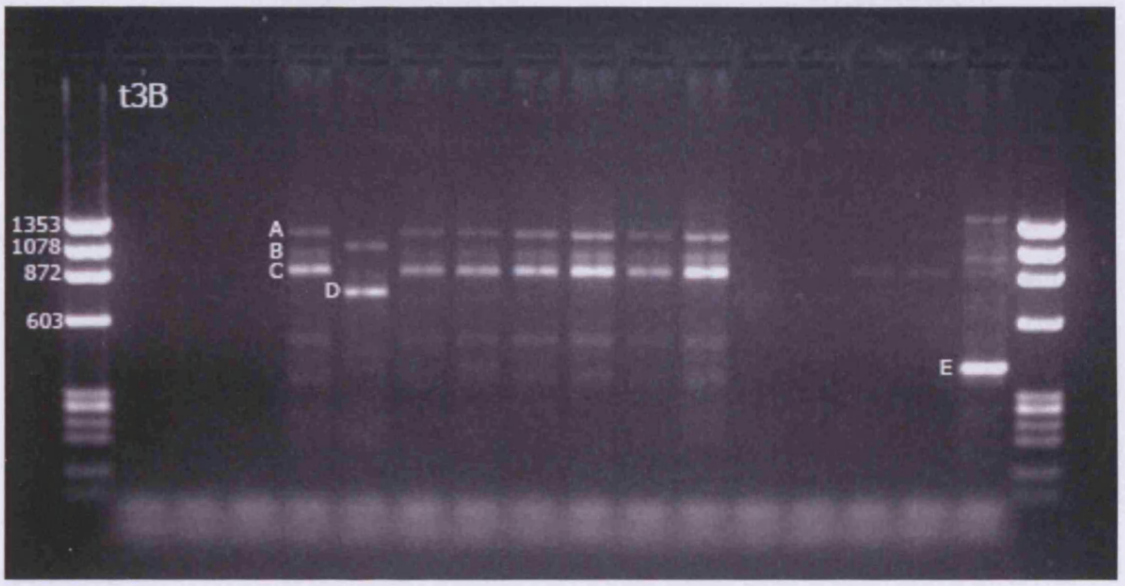

Figure 7.3: Examples of electrophoretic separation of PCR fingerprinting using T3 B sequence

Five bands were selected for evaluation in this study: A: ~1053 bp; B: ~1078 bp; C: ~872 bp; D: below 872 bp; E: above 310 bp

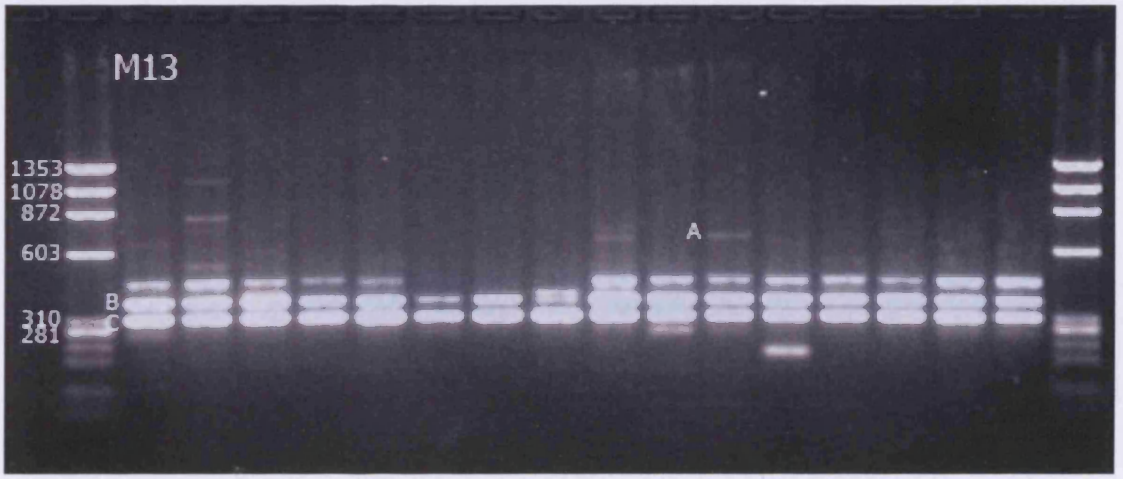

Figure 7.4: Examples of electrophoretic separation of PCR fingerprinting using M13 phage core sequence

Three bands were selected for evaluation in this study: A: below 872 bp; B: above 310 bp; C: ~310 bp

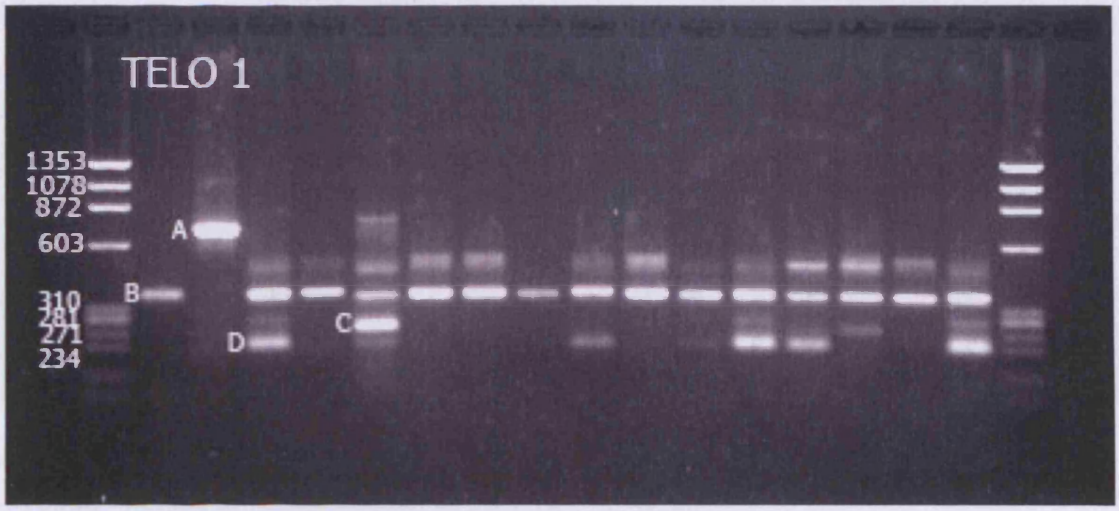

Figure 7.5: Examples of eletrophoretic separation of PCR fingerprinting using TELO 1

Four bands were selected for evaluation in this study: A: above 603 bp; B: above 310 bp; C: ~271 bp; D: ~234bp

Table 7.3: Statistical analysis of the PCR fingerprint banding pattern

Variable	Isolates	N	Mean	p value
All isolates	89	3916	3.24 (2.06)	
Genotype A	60	1653	3.72 (2.25)	
Genotypes B and C	29	465	2.23 (1.17)	**<0.0001#**
Non-DM control subjects	29	406	3.09 (1.79)	
UK DM patients	30	435	3.65 (2.28)	
Italian DM patients	30	435	2.93 (1.92)	**<0.0001***

Statistical analysis was performed using T test (#) and ANOVA (*) test
N is the number of comparisons made between the isolates within the cohort of patients resulting in the given mean (SD = standard deviation) genotypic diversity for each of the groups

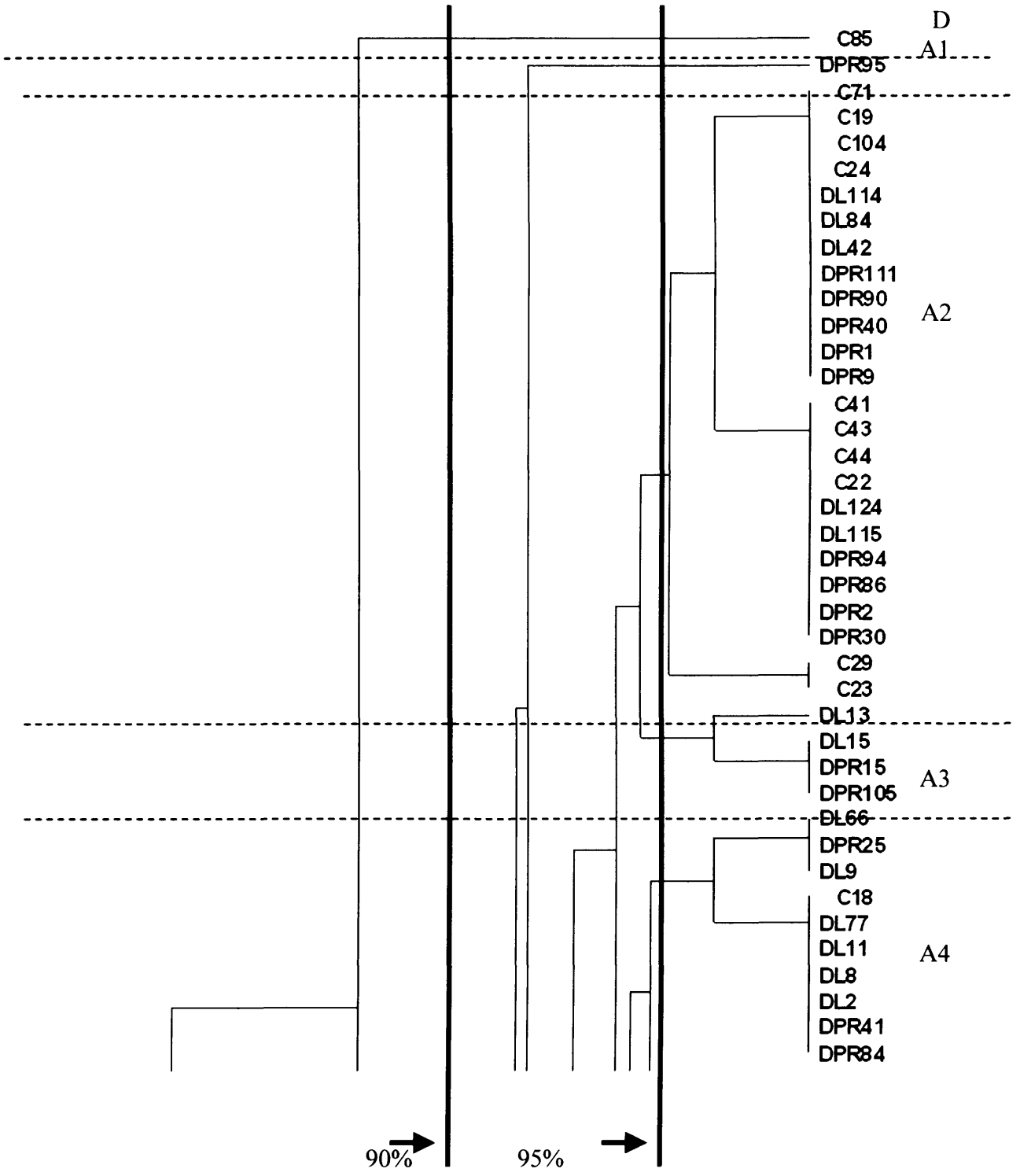

Figure 7.6-A: First part (of two) of the phylogram of the 89 *C. albicans* isolates based on the analysis of the PCR fingerprinting results

Horizontal lines are shown, strains to the right of these are 90% and 95% similar, vertical lines and designations shown on the right margin are based on these levels of similarity and are listed in Table 7.4

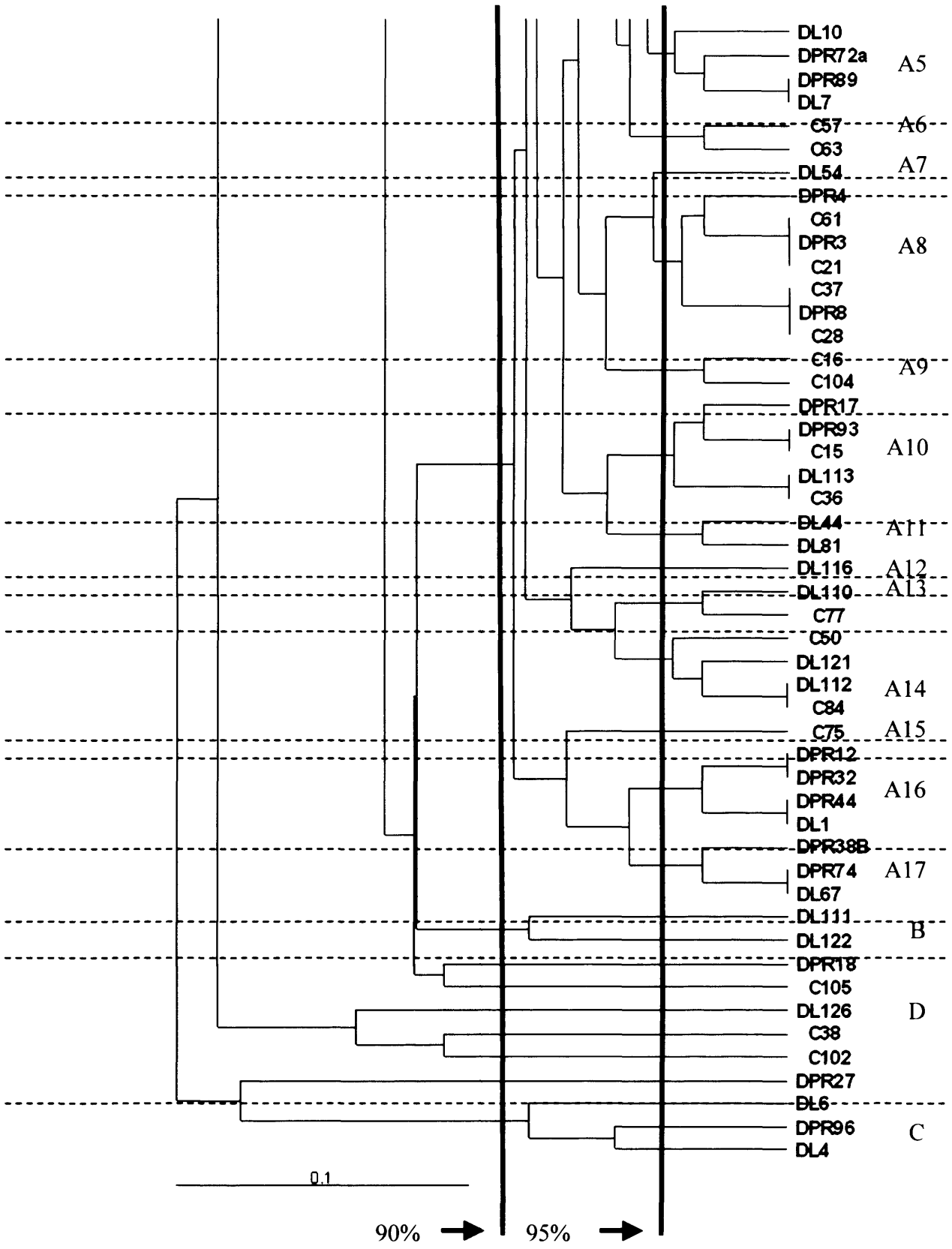

Figure 7.6-B: Second part (of two) of the phylogram of the 89 *C. albicans* isolates based on the analysis of the PCR fingerprinting results

Horizontal lines are shown, strains to the right of these are 90% and 95% similar, vertical lines and designations shown on the right margin are based on these levels of similarity and are listed in Table 7.4

Table 7.4: Genotypic distribution of the 89 *C. albicans* isolates based on phylogenetic analysis of the PCR fingerprinting results

Genotype group	Isolate designation
A1	DPR95
A2	CM71, CM19, CM104, C24, DL114, DL84, DL42, DPR111, DPR90, DPR40, DPR1, DPR9, CZ41, CZ43, CZ44, CZ22, DL124, DL115, DPR94, DPR86, DPR2, DPR30, CM29, CM23
A3	DL13, DL15, DPR15, DPR105
A4	DL66, DPR25, DL9, CM18, DL77, DL11, DL8, DL2, DPR41, DPR84
A5	DL10, DPR72a, DPR89, DL7
A6	CZ57, CZ63
A7	DL54
A8	DPR4, CZ61, DPR3, CZ21, CZ37, DPR8, CZ28
A9	CZ16, CZ104
A10	DPR17, DPR93, CZ15, DL113, CZ36
A11	DL44, DL81
A12	DL116
A13	DL110,CM77
A14	CZ50,DL121,DL112,CM84
A15	CM75
A16	DPR12, DPR32,DPR44,DL1
A17	DPR38b,DPR74,DL67
B	DL111, DL122
C	DL6,DPR96,DL4
D	CM85, DPR18, CZ105, DL126, CZ38, CZ102, DPR27

The three large genotypic groups (A to C) are isolates that are co-located on the phylogram (Figure 7.6) and are more than 90% genotypically similar, while the other 17 genotypic subgroups (A1-A17) are co-located and more than 95% similar (see phylogram Figure 7.6). The final genotypic group, Group D, consists of the most diverse isolates; none of these isolates are more than 90% similar to one another or to any other isolate. The letter in the isolate designation indicates the patient cohort from which the isolate was isolated: CM and CZ = non-DM control patients; DL = DM patients from London UK; DPR = DM patients from Parma, Italy

7.4 Discussion

The observed variation in genotypic diversity of the *C. albicans* isolates in the present study may be indicative of the distinct ecological niches that occur in the oral cavities of these patient cohorts. The statistical correlation of this genotypic diversity, as assessed in the present study, shows that the group of isolates isolated from the DM patients in the UK were the most diverse (mean diversity = 3.65, Table 7.3). This diversity was statistically higher than both control patient isolates (mean diversity = 3.09, Table 7.3) and isolates from DM patients in Italy (mean diversity = 2.93, Table 7.3). This result may reflect the patient cohort from which these strains were isolated, with DM patients in the UK affected by long-standing DM and with more diabetic complications (neuropathies and retinopathies) than patients from Italy. Alternatively, it may reflect the antifungal treatment that these patients received. As reported in the previous Chapter (Section 6.3.4), more *Candida* isolates from UK patients had increased resistance to azole agents and a trend of increased resistance to amphotericin B than those from DM patients from Parma.

Further research is necessary to elucidate the true cause of the observed genotypic diversity, with the inclusion of isolates from patient cohorts with systemic diseases different to DM, and from more diverse geographic localities.

The broad genotypic subgroups of isolates designated as subgroup A, B and C (Section 2.5.1) are based on the presence or absence of a transposable intron within the 25 S rDNA (Mercure *et al*, 1993). Genotypic subgroup A does not have this intron, while in subgroup B it is present in all copies of the 25 S rDNA and in subgroup C it is partially present (Mercure *et al*, 1993). The analysis of the genotypic diversity in the present study showed that subgroup A isolates without the intron are less genotypically cohesive than sub-group B or C isolates. It may well be that the ability to acquire this intron within the 25 S rDNA is confined to a particularly

genetically cohesive sub-group of *C. albicans* that possess currently unknown phenotypic traits. Interestingly, the present study did not find any association between a particular genotype of *C. albicans* and the type, disease progression and degree of control of DM (Chapter 4, Section 4.3.1.3), although previous studies have shown that these strains exhibit enhanced antifungal susceptibility (Mercure *et al*, 1993). Phylogenetic analysis of these isolates, as presented in the phylogram (Fig. 7.6, A and B) and the table of clustered isolates (Table 7.4) shows a different aspect of the genotypic diversity of these isolates from that expressed by the statistical analysis. The statistical analysis describes the similarity that each isolate had with other isolates from patients within the same cohort. The phylogenetic analysis assesses the uniqueness of each isolate and parsimoniously compares this with all isolates studied. Phylogenetic analysis has revealed several interesting findings. Firstly, this PCR fingerprinting method found genetic identity in eight pairs of isolates (subgroups A2, A5, A10 twice, A14, A16 twice and A17), four triplets (subgroups A3, A4 and A8 twice), a group of 7 isolates (subgroup A4), a group of 10 isolates (subgroup A2) and a group of 12 isolates (also in subgroup A2) (Fig. 7.6). This would indicate that the PCR fingerprinting method is not so overtly sensitive that it discriminates all isolates to the individual level, yet is able to cluster genetically similar isolates into useful epidemiological subgroups. Furthermore, the fact that all 17 PCR fingerprinting bands chosen for inclusion in the analysis were parsimony-informative reflects the genetic diversity of these codominant markers.

Secondly, out of the 15 groups of isolates outlined in the preceding paragraph that showed genotypic identity with this method, in only two of these groups did all isolates come from the same patient cohort. This occurred with two pairs of isolates in the subgroups A2 and A16. The fact that this PCR fingerprinting method found isolate identities between isolates from all three patient cohorts reflects the genetic

similarity that exists within this fungal species. A more interesting observation is the extreme genetic diversity seen in isolates within subgroup D (Fig. 7.6 and Table 7.4). These isolates had very little genetic relationship with either the other *C. albicans* isolates or other members of the same subgroup. The presence of these isolates did not skew the statistical analysis (Table 7.4) as only one of these 7 isolates (DL126) came from the most genotypically diverse patient cohort (the strains isolated from the DM patients in London, UK). Therefore, as indicated above there must be other reasons for the genotypic diversity observed in the DM London patient isolates. Nevertheless, these extremely genetically diverse isolates that make up this subgroup D (Fig. 7.6 and Table 7.4) warrant further study. It could be hypothesised that this subgroup represents a unique non-clonal expansion of *C. albicans* strains that may indicate either sexual or some other method of rapid transfer of genetic information.

Finally, and as indicated above, the present analysis was only conducted with patients from two localities, who were either deemed suitable to represent a "normal" control population, or who had DM.

Further studies that include isolates from patient cohorts with systemic diseases other than DM, and from more diverse geographic localities are needed in order to compare the results obtained in these population groups.

CHAPTER 8

In vitro activity of a rat monoclonal IgM antibody (K10) and of a synthetic anti-idiotype peptide (KP) against different strains of *Candida* spp.

8.1 Introduction

Yeast killer toxins (KTs) are exotoxins (generally proteins or glycoproteins) secreted by a number of yeast species that are capable of killing susceptible cells belonging to the same or different species (Somers & Bevan, 1969; Young & Yagiu, 1978; Wickner, 1985; Polonelli *et al*, 1991b; Magliani *et al*, 1997b). Killer cells are immune to their own toxins, and much remains to be elucidated over the mechanisms of killing and immunity associated with killer systems (Magliani *et al*, 1997b).

Interest in the yeast killer phenomenon has recently increased, particularly because of the surprising susceptibility of microorganisms of clinical interest, such as *C. albicans*, *Pneumocystis carinii* and *Mycobacterium tuberculosis*, to killer toxins from species of the genera *Pichia* and *Williopsis* (Polonelli & Morace, 1986a; Morace *et al*, 1989; Aliouat *et al*, 1993; Magliani *et al*, 1997b).

A toxin produced by the killer yeast *Pichia anomala* ATCC 96603 (PaKT) has been identified as a large glycopeptide molecule and exhibits activity against organisms possessing target cell-wall receptors. *Candida* species are among the organisms susceptible to PaKT and to this effect the β-glucans of the cell wall are believed to act as toxin receptors (Polonelli & Morace, 1986b; Polonelli *et al*, 1990). However, even if the candidacidal activity of PaKT is mediated in part, or as a whole by its

interaction with β-glucans, the exact mechanism through which PaKT works is still unknown.

A wide spectrum of activity has led to the consideration of a potential therapeutic effect of PaKT (Polonelli *et al*, 1986) in the treatment of fungal infections. Regretfully, the value of PaKT as an antibiotic therapeutic is hampered due to its high level of toxicity, antigenicity and its instability at physiological pH (Pettoello-Mantovani *et al*, 1995; Conti *et al*, 1996). Anaphylactic reactions may be provoked, or neutralizing antibodies may be produced by the immune system after administration of yeast killer toxins. Only small biologically active killer peptides, devoid of immunogenicity, have the potential to be applied therapeutically (Magliani *et al*, 1997b).

In order to exploit the antimicrobial activity of PaKT without undesired side-effects, a KT-neutralizing monoclonal antibody (mAbKT4) has been generated according to the idiotypic network theory (Jerne, 1974), for the production of anti-idiotypic antibodies, and this antibody mimics the killer toxin, PaKT.

The idiotypic network is based on the dual character of the antibody molecule, in which any antibody is able to interact with an antigen through the antigen-binding site (paratope) and at the same time, its variable region (idiotope) can be matched by a complementary three-dimensional surface of another antibody variable region (anti-idiotype). An idiotype produced in the course of an immune response against a particular antigen can stimulate the production of complementary anti-idiotypes, according to an idiotypic cascade (Figure 8.1) (Magliani *et al*, 1997b). Production of anti-idiotypes may be self-limiting because of their similarity to the idiotypes. An important implication of the network theory is that the combining site of some anti-idiotypes, which are complementary to the antigen-complementary idiotype, may resemble the immunogenic determinant of the external antigen (Figure 8.1, Ab2β). In

this case, the anti-idiotype variable region represents the internal image of the external antigen and, in certain circumstances, may mimic its biological activity (Georgatos, 1993; Fields *et al*, 1995).

According to the theory of the idiotypic network, mAbKT4 was used as immunogen (idiotypic vaccination) to elicit anti-idiotypic antibodies representing the internal image of PaKT. Immunisation of animals of different species (rabbits, rats and mice) by intravenous or intravaginal route with mAb KT4 resulted in the production of serum or secretory immunoprotective KT-like anti-idiotypic antibodies (KT-IdAb) (Polonelli & Morace, 1988; Polonelli *et al*, 1991a; Polonelli *et al*, 1993; Polonelli *et al*, 1994).

KT-IdAb, as an internal image of PaKT, has been shown to interact with putative killer toxin cell-wall receptors (KTR) of sensitive *C. albicans* cells (Polonelli *et al*, 1990; Magliani *et al*, 1997b). KTR has recently been identified as having β-glucan moieties (Guyard *et al*, 2002a; Guyard *et al*, 2002c) thus indirectly supporting the concept that KT-IdAb could indeed recognize the β-glucan cell-wall polysaccharide components through which it could exert its cytocidal action (Cassone *et al*, 1997).

The KT-IdAb, which contains the internal image of KT and thus mimics PaKT's fungicidal effect, has demonstrated killer activity against KT-sensitive yeasts (Magliani *et al*, 1997a). To obtain standardizable reagents in sufficient amounts for therapeutic assays, KT-IdAb were produced in the monoclonal (KtmAb-K10) and recombinant single-chain fragment variable (KTscFvH6) format by hybridoma and phage display molecular technologies from animals vaccinated with mAbKT4 (Magliani *et al*, 1997a; Polonelli *et al*, 1997). All these novel KT-mimicking antibodies displayed *in vitro* and *in vivo* antimicrobial activity against KTR possessing, pathogenic microorganisms (Magliani *et al*, 1997a; Seguy *et al*, 1997; Conti *et al*, 1998).

Does KT-IdAb have reduced [illegible] compared with KT?

In an attempt to identify biologically active fragments for possible therapeutic use, the entire sequence of KTscFvH6 was sequenced and synthesised in a number of overlapping decapeptides, each displaced of two residues, and with special focus on those pertaining to complementary-determining region (CDR) domains. A synthetic decapeptide fragment (P6), which was derived from the sequence of the variable region of KTscFvH6 and contained part of the CDR1 of the VL chain, was analysed by alanine scanning in order to identify the functional contribution of each residue (Section 2.10). The decapeptide with the highest candidacidal activity (KP) was then selected for large scale synthesis based on its high candidacidal reactivity *in vitro.* Figure 8.2 shows the interaction of PaKT with KTR and also that of natural, anti-idiotypic polyclonal, monoclonal and recombinant antibodies, representing its internal image, and synthetic peptides (mimotopes) (Magliani *et al*, 2004).

Aim: The aim of this study was to evaluate the killer activity of a monoclonal anti-idiotypic antibody (K10) and of the synthetic decapeptide (KP) on selected *Candida* spp. isolated from the oral cavity of DM patients and a non-DM control population.

Hypothesis: Differences occur in *Candida* isolates susceptibility to mAbK10 and KP depending on their patient origin and that these differences correlate with the susceptibility profiles observed using conventional antifungal agents.

8.2 Methods

8.2.1 mAbK10

Hybridoma cells secreting mAbK10 were grown in a specific medium (Section 2.9.1) and the supernatant from the culture was collected, precipitated in ammonium

sulphate saturated solution and then dialysed against PBS for 48 h (Section 2.9.1) (Polonelli *et al*, 1997).

Antibody concentration was achieved by capture ELISA using a pair of mouse monoclonal antibodies against μ heavy chain of rat immunoglobulin (Section 2.9.1).

The candidacidal activity of mAbK10 was evaluated *in vitro* by a cfu assay on selected isolates of *Candida* spp. from each of the patient group's investigated in this study (DM patients from London and Parma and a control non DM group, Sections 2.1.1, 2.1.2, 2.1.3). Due to the limited availability of K10 and KP, analysis was undertaken with a reduced but still representative number of *Candida* isolates.

Twelve *Candida* isolates (*C. albicans* and non-*C. albicans* spp.) that had previously been shown to be sensitive to six antifungal agents (Section 2.8 and 6.3) and a further 12 resistant isolates were initially selected from each patient group for this study. However, during the course of investigation one of the isolates (from a DM patient from Parma) did not re-culture and was excluded. Time constraints precluded its replacement (Tables 8.1-8.2).

Statistical analysis of data was performed using the Mann-Whitney (when two groups of data were analysed) or Kruskall-Wallis (when more than two groups of data were analysed) non parametric tests and differences within or between groups were considered significant when the probability (p) was less than or equal to 0.05.

8.2.2 Killer Peptide (KP)

Soluble powder of synthesised KP was kindly provided by Professor Polonelli (Sezione di Microbiologia, Dipartimento di Patologia e Medicina di Laboratorio, Parma University, Italy). The candidacidal activity of KP was evaluated *in vitro* by a cfu assay on selected isolates of *Candida* spp. as detailed earlier (Section 2.10).

8.3 Results

8.3.1 Candidacidal activity of mAbK10

The rat monoclonal mAbK10 was found to have a significant antifungal activity against all the *Candida* strains evaluated in this study (Tables 8.1-8.2-8.3).

To confirm that the colonies of *Candida* spp. that initially survived the killer activity of mAbK10 were not resistant mutants of *Candida* strains, a second cfu assay was performed on these colonies under the same experimental conditions. All isolates remained sensitive with a 100% killing rate upon repeat testing.

No significant differences (p=0.45) were observed in the candidacidal activity of mAbK10 between *C. albicans* (50-99% killing rate, mean = 76.9, SD = 13.1) and non-*C. albicans* isolates (30 –99.5% killing rate, mean = 73.2, SD = 17.8) (Table 8.1). In addition, no significant differences (p=0.76) were evident in the candidacidal activity of mAbK10 against oral yeast isolates that had previously found to be either resistant or intermediately resistant to conventional antifungals (30-99.5% killing rate, mean = 74, SD = 17) and those isolates that were previously susceptible (50-97% killing rate, mean = 76.1, SD = 13.8) (Table 8.2). Furthermore, no statistically significant differences (p=0.71) were again apparent in the candidacidal activity of mAbK10 for the isolates from the different patient populations (London DM patients, 45-99% killing rate, mean = 77.5, SD = 12.8; Parma DM patients, 30-99.5% killing rate, mean = 72.7, SD = 18.4; and non-DM controls (45-99% killing rate, mean = 74.9, SD = 15) (Table 8.3).

8.3.2. Candidacidal activity of KP

Synthetic peptide KP had a significant candidacidal activity upon all the *Candida* isolates examined in this study. There was a 100% killing rate when a concentration

of 100μg/ml KP was used. Although the killing rate decreased when a concentration of 20μg/ml was used, although for most isolates the killing rate still exceeded 90% (Tables 8.1-8.2-8.3).

To confirm that the colonies of *Candida* spp. that initially survived the killer activity of KP were not resistant mutants of *Candida* strains, a second cfu assay was again performed under the same experimental conditions and as previously noted with mAbK10, all the isolates were sensitive.

No significant differences ($p=0.14$) were evident in the candidacidal activity of KP for *C. albicans* (58-100% killing rate, mean = 92.8, SD = 10.1) and non-*C. albicans* isolates (68.7–100% killing rate, mean = 92.2, SD = 7.1) (Table 8.1). In addition, there were no differences ($p=0.47$) in the candidacidal activity of KP against oral yeast isolates that had previously been shown to be either resistant or intermediately resistant to conventional antifungals (61-100% killing rate, mean = 93, SD = 8.5) and those isolates that were previously susceptible (58-100% killing rate, mean = 92.1, SD = 8.7) (Table 8.2).

Statistically significant differences ($p<0.01$) were observed in the antifungal activity of KP against the isolates from London DM patients (58-100% inhibition, mean = 89.9, SD = 11.4) and those from Parma (84-100% inhibition, mean = 96.2, SD = 5.3) (Table 8.3). Statistically significant differences ($p<0.01$) were also observed in the antifungal activity of KP against isolates from Parma DM patients (84-100% inhibition, mean = 96.2, SD = 5.3) and non DM controls (75-100% inhibition, mean = 91.7, SD = 6.7) (Table 8.3).

The fungicidal action of KP was dose-dependant, as determined by testing the peptide at different concentrations against small groups of *C. albicans* and non-*C. albicans* isolates of differing antifungal susceptibilities. Almost 100% fungicidal activity was

obtained at 20μg/ml of KP, but good candidacidal activity was also evident at 15μg/ml (90-99% *Candida* killing rate) and at 10μg/ml (48-96% *Candida* killing rate) of KP. At a concentration of 5μg/ml, the candidacidal action of KP showed considerable variation and with one *C. albicans* isolate expressing resistance to itraconazole, 5μg/ml of KP had no candidacidal action (Table 8.4).

8.4 Discussion

Recently, rat IgM monoclonal (mAbK10) and recombinant single-chain fragment variable (scFvH6) anti-idiotypic antibodies, functionally mimicking the activity of PaKT, have been produced by idiotypic vaccination with a mouse monoclonal antibody (mAbKT4) that neutralised PaKT (Magliani *et al*, 1997a; Polonelli *et al*, 1997). Monoclonal antibody K10 and scFvH6 displayed microbicidal activity *in vitro* and a therapeutic effect *in vivo* against KTR-bearing eukaryotic and prokaryotic pathogenic microorganisms (Magliani *et al*, 1997a; Seguy *et al*, 1997; Conti *et al*, 1998; Conti *et al*, 2000; Cenci *et al*, 2002; Conti *et al*, 2002; Savoia *et al*, 2002). The most active among the synthesised decapeptides of scFvH6 (P6), containing part of the CDR-L1 region, was selected for large scale synthesis and analysed through alanine scanning to evaluate the critical relevance of each residue. A decapeptide (KP) proved, in comparison with its own scramble peptide (SP), to exert enhanced candidacidal activity *in vitro*. This activity was found to be inhibited by laminarin (a soluble β–1,3 glucan preparation) but not by pustulan (a soluble β–1,6 glucan preparation). Furthermore, KP also showed a potent therapeutic effect *in vivo* in immunocompetent and immunosuppressed mice against vaginal and systemic candidiasis caused by fluconazole sensitive and resistant strains (Polonelli *et al*, 2003).

In the present study, mAbK10 and KP have been shown to have significant antifungal activity on a wide spectrum of oral isolates of different *Candida* spp., irrespective of the species or previous susceptibility to conventional antifungals. However, KP activity on the *Candida* isolates from Parma DM patients was higher than that of isolates from the London DM patients ($p=0.006$) and that of the non-DM control subjects ($p=0.02$). This observed variation is not readily explained, but may reflect the natural variability of expression of the KTR throughout all fungi. It has been reported (Chapter 6, Section 6.3.4) that the isolates from the London DM population were more resistant to several conventional antifungal agents than *Candida* isolates from Parma and from the control group. Furthermore, although the range of KP activity on the isolates of the three groups was varied, the inhibition of candidal growth by KP on the isolates of the London DM group was over 58% (Table 8.3), even for the isolates that were completely resistant to the conventional antifungal agents tested. The wide spectrum of candidacidal activity most likely reflects the ability of mAbK10 and KP to interact with all tested β-glucan-KTR-possessing pathogenic microorganisms, including *Candida* spp. (Magliani *et al*, 1997a; Polonelli *et al*, 1997; Conti *et al*, 1998). It has recently been described (Polonelli *et al*, 2003) that the candidacidal activity of mAbK10 and KP against the reference *C. albicans* strain was inhibited dose-dependently by laminarin (a β–1,3 glucan soluble product representing the main target of PaKT). Furthermore, it has recently been reported that the KTR is a β-glucan component of the cell wall (Guyard *et al*, 2002b; Guyard *et al*, 2002c). As glucan and glucan-like molecules are widely present in microbes, where they exert critical structural and/or virulence properties, their targeting may provide a novel and extensive therapeutic approach to microbial infections (Bromuro *et al*, 2002). Therefore, mAbK10 and KP can display a wide spectrum of antimicrobial activity, including yeasts and filamentous fungi, other than *Candida* spp., and this

activity has been shown in diverse species such as *Aspergillus fumigatus* (Cenci *et al*, 2002), *Cryptococcus neoformans* (Cenci *et al*, 2004), *Paracoccidioides brasiliensis*, *Pneumocystis carinii* (Seguy *et al*, 1997), as well as protozoa, such as *Leishmania* spp. (Savoia *et al*, 2002) and bacteria (Conti *et al*, 1998; Conti *et al*, 2000; Conti *et al*, 2002).

The previously described variation in the susceptibility of the different yeast isolates to mAbK10 and KP may reflect the expression of specific cell-wall receptors. *Candida* colonies that initially survived exposure to mAbK10 and KP during the cfu assay were subsequently shown to be equally susceptible in a second cfu experiment (*i.e.* they were not resistant mutants of *Candida* strains). Changes in the time of interaction of the *Candida* cells with mAbK10 and KP or in the different phase of growth of *Candida* cells were shown to influence the killing activity on the same *Candida* isolates, presumably in terms of expression of number of cell-wall receptors that could interact with the sufficient amount of mAbK10 and KP (Polonelli *et al*, 2003).

The marked susceptibility of strains of *Candida* spp. (resistant to conventional antifungal agents) to mAbK10 and KP is of particular interest in view of increased clinical concern regarding antimicrobial resistance in these and allied fungi (Alexander & Perfect, 1997). The mechanisms of resistance to conventional antifungal agents found in the *Candida* spp. isolates investigated in the present study do not affect the susceptibility of these isolates to mAbK10 and KP. Even though the active mechanism of these antibody derivates is still undetermined, their evident interaction with a presumptive KTR localized in the cell wall, such as β-glucan, suggests the presence of a fungal target that has therapeutic potential. This may be similar to a β-1,3 glucan synthesis inhibitor, such as pneumocandin or echinocandin, which displays fungicidal activity on different species of yeast and mould (Onishi *et*

al, 2000; Denning, 2003). Resistance to these antifungal agents has been reported in spontaneous mutants of *S. cerevisiae* and *C. albicans*. However, this resistance may not be of clinical relevance as the mutants exhibit attenuated virulence in animal models of experimental infections (Douglas *et al*, 1994; Kurtz *et al*, 1996).

KP exerted greater candidacidal activity on all the isolates tested in the study (percentage of *Candida* killing rate, total mean = 92.5) than mAbK10. This may reflect the different structure of the two tested molecules compared with the same concentration (20μg/ml) but characterised by different molecular weights (KP = ~1 Kda, K10 = ~900 Kda) where K10 is a rat monoclonal Ig M antibody (Polonelli *et al*, 1997), and KP a synthetic decapeptide containing the first three amino acids of the light chain CDR1, which represents an active fragment of the KTscFv. Interestingly, the deletion of the COOH-terminal serine alone caused a remarkable drop (about three logs) in KP activity, as recently reported (Polonelli *et al*, 2003). Nevertheless, the engineered synthetic anti-idiotype fragment KP represents the evolution of KT-IdAbs, through the monoclonal (K10) and recombinant format (Magliani *et al*, 1997a; Magliani *et al*, 1997b).

The impressive candidacidal activity of KP *in vitro* at low concentrations (5-10μg/ml) on four different selected *Candida* strains, indicates that KP may be capable of exerting antifungal action *in vivo* with therapeutic administration. An *in vivo* experimental model of rat vaginal candidosis showed that KP was capable of significantly accelerating the clearance of *Candida* from the vagina of rats, similar to a therapeutic course of fluconazole (De Bernardis *et al*, 1999a). KP also eradicated the vaginal infection in rats caused by a fluconazole-resistant strain of *C. albicans*, a finding of special interest in view of increased clinical concern regarding fluconazole resistance in this and allied fungi (Edwards, 1991; Alexander & Perfect, 1997).

The potent therapeutic effect of KP was also observed in a mouse model of systemic candidosis (Mencacci *et al*, 1994) where the curative effect was similarly exerted in normal, immunocompetent Balb/c as well as in SCID mice, demonstrating that the therapeutic benefit of KP did not require the participation of an adaptive host immunity (Polonelli *et al*, 2003).

Although both mAbK10 and KP expressed good candidacidal activity in this *in vitro* study, KP in particular, as a synthetic peptide, may represent the compound for the generation of a new class of broad-spectrum antimicrobial molecules for the prevention and treatment of superficial and systemic infection caused by KT-sensitive microorganisms. The mechanism of action of this new class of drug reflects a naturally occurring phenomenon, that of killer yeasts, and as such may be able to overcome currently recognised mechanisms of resistance to conventional antimicrobial drugs in epidemiologically relevant prokaryotic and eukaryotic pathogenic microorganisms.

Table 8.1: Susceptibility of *C. albicans* and non-*C. albicans* isolates to mAbK10 and KP

C. albicans isolates	K10[a] (%)	KP[a] (%)	*Candida* isolates	K10[a] (%)	KP[a] (%)
C. albicans A DL 2	99	100	*C. dubliniensis* DL 63	90	91.7
C. albicans A DL 4	97.1	97.7	*C. dubliniensis* DL 41	97	89.7
C. albicans A DL 7	77.4	98.6	*C. dubliniensis* DL 92	75.1	92
C. albicans A DL 1	83.4	97.9	*C. dubliniensis* DL 72	70	68.7
C. albicans A DL 18	71	93.1	*C. dubliniensis* DPR 47	73.4	99
C. albicans A DL 24	70.5	89.8	*C. dubliniensis* CZ 69	65.2	75.3
C. albicans A DL 66	84.1	92.2	*C. dubliniensis* CM 24	93.1	88.2
C. albicans A DL 121	78.2	58.2	*C. dubliniensis* CM 31	96.1	95.6
C. albicans A DPR 59	65	99.7	*C. dubliniensis* CM 35	70	98.1
C. albicans A DPR 67	50.1	100	*C. dubliniensis* CM 72	60.3	92.5
C. albicans A DPR 96	74.3	97.4	*C. glabrata* DL 48	73.4	96.3
C. albicans A DPR 98	65.9	98.4	*C. glabrata* DL 102	65	83.3
C. albicans A DPR 100	90.4	100	*C. glabrata* DL 107	51.6	99.5
C. albicans A DPR 38a	52.7	82.9	*C. glabrata* DL 125	93	95.9
C. albicans A DPR 39	62.4	98	*C. glabrata* DPR 28	99.5	84.4
C. albicans A DPR 95	80	97.1	*C. glabrata* DPR 33	82	96.9
C. albicans A DPR 107	97	100	*C. glabrata* DPR 24	62.1	90.5
C. albicans A CZ 58	99	92.4	*C. glabrata* CZ 96a	45.4	95.4
C. albicans A CM 75	70.5	76.9	*C. glabrata* CZ 20	80	87.2
C. albicans A CZ 22	74.4	97.1	*C. glabrata* CZ 98	54.1	96.3
C. albicans A CM 76	92.2	93.4	*C. guilliermondii* DL 73	78.4	95.2
C. albicans A CM 95	60	100	*C. guilliermondii* CZ 29	94.4	92.2
C. albicans B DL 67	78.3	61	*C. krusei* DL 70	45	94.2
C. albicans B DL 13	77	92.9	*C. krusei* CM 30b	56.1	95.1
C. albicans B DPR 89	76.2	98	*C. krusei* CZ 96b	87	79.4
C. albicans B DPR 111	55.3	99	*C. krusei* CM 20	60	94
C. albicans B CM 104	80.2	99.3	*C. parapsilosis* DL 98	72.1	93
C. albicans B CM 23	85.3	84.7	*C. parapsilosis* DPR 66	30	99.3
C. albicans C DL 54	80.2	90.5	*C. parapsilosis* DPR 23	93.4	94.1
C. albicans C DL 84	75	87.2	*C. parapsilosis* DPR 68	88	99.2
C. albicans C DPR74	87.4	97.8	*C. tropicalis* DL 86	78.4	100
C. albicans C DPR 9	91.4	99.8	*C. tropicalis* DPR 51	50	84
C. albicans C CM 29	77	90.1	*C. lusitaniae* DPR 31	91.1	98.8
C. albicans C CM 110	60	96.7	*C. lusitaniae* DPR 65	56.4	98.7
			C. lusitaniae CM 30a	86.2	93.7

[a]mAbK10 and KP were used at a concentration of 20 μg/ml; their activity is expressed as a percentage killing rate evaluated by the cfu assay in comparison with the proper controls
Statistical analysis was performed using the Mann-Whitney non parametric test
DPR: DM patients from Parma, Italy; DL: DM patients from London, UK; CM and CZ: non-DM subjects

Table 8.2: mAbK10 and KP candidacidal activity on *Candida* isolates with different antifungal susceptibilities

Candida isolates	K10[a] (%)	KP[a] (%)	AS[b]	*Candida* isolates	K10[a] (%)	KP[a] (%)	AS[b]
C. albicans A DL 2	99	100	itr^R	*C. albicans* A DL 1	83.4	97.9	S
C. albicans A DL 4	97.1	97.7	mcz^I	*C. albicans* A DL 18	71	93.1	S
C. albicans A DL 7	77.4	98.6	itr^R ket^R $5\text{-}FC^R$	*C. albicans* A DL 24	70.5	89.8	S
C. albicans B DL 67	78.3	61	ket^I mcz^I flu^R itr^R	*C. albicans* A DL 66	84.1	92.2	S
C. albicans C DL 54	80.2	90.5	mcz^I	*C. albicans* A DL 121	78.2	58.2	S
C. dubliniensis DL 63	90.0	91.7	flu^I mcz^I	*C. albicans* B DL 13	77	92.9	S
C. glabrata DL 48	73.4	96.3	ab^I flu^I itr^I mcz^I	*C. albicans* C DL 84	75	87.2	S
C. glabrata DL 102	65.0	83.3	mcz^I flu^R ket^R itr^R	*C. dubliniensis* DL 41	97.0	89.7	S
C. glabrata DL 107	51.6	99.5	flu^I ket^I itr^I mcz^I	*C. dubliniensis* DL 92	75.1	92.0	S
C. guilliermondii DL 73	78.4	95.2	ket^I itr^I mcz^I	*C. dubliniensis* DL 72	70.0	68.7	S
C. krusei DL 70	45.0	94.2	flu^I mcz^I itr^R	*C. glabrata* DL 125	93.0	95.9	S
C. parapsilosis DL 98	72.1	93	flu^I mcz^I $5\text{-}FC^I$	*C. tropicalis* DL 86	78.4	100	S
C. albicans A DPR 59	65	99.7	itr^I	*C. albicans* A DPR 38a	52.7	82.9	S
C. albicans A DPR 67	50.1	100	$5\text{-}FC^R$	*C. albicans* A DPR 39	62.4	98	S
C. albicans A DPR 96	74.3	97.4	itr^I	*C. albicans* A DPR 95	80	97.1	S
C. albicans A DPR 98	65.9	98.4	ket^I flu^R itr^R	*C. albicans* A DPR 107	97	100	S
C. albicans A DPR 100	90.4	100	itr^I	*C. albicans* B DPR 111	55.3	99	S
C. albicans B DPR 89	76.2	98	ket^I	*C. albicans* C DPR 9	91.4	99.8	S
C. albicans C DPR74	87.4	97.8	itr^I	*C. dubliniensis* DPR 47	73.4	99.0	S
C. glabrata DPR 28	99.5	84.4	flu^I itr^I mcz^I	*C. glabrata* DPR 24	62.1	90.5	S
C. glabrata DPR 33	82.0	96.9	itr^I	*C. parapsilosis* DPR 23	93.4	94.1	S
C. lusitaniae DPR 31	91.1	98.8	flu^I ket^I itr^I mcz^I $5\text{-}FC^I$	*C. parapsilosis* DPR 68	88.0	99.2	S
C. lusitaniae DPR 65	56.4	98.7	flu^I ket^I itr^I mcz^I $5\text{-}FC^I$	*C. tropicalis* DPR 51	50.0	84.0	S
C. parapsilosis DPR 66	30.0	99.3	ket^I	-	-	-	-
C. albicans A CZ 58	99	92.4	itr^R	*C. albicans* A CZ 22	74.4	97.1	S
C. albicans A CM 75	70.5	76.9	ab^I mcz^I flu^R itr^R ket^R	*C. albicans* A CM 76	92.2	93.4	S
C. albicans B CM 71	70.2	94.4	ket^I itr^R	*C. albicans* A CM 95	60	100	S
C. albicans B CM 104	80.2	99.3	mcz^I	*C. albicans* B CM 23	85.3	84.7	S
C. albicans C CM 29	77	90.1	$5\text{-}FC^I$	*C. albicans* A CZ 11	81.4	94.4	S
C. dubliniensis CZ 69	65.2	75.3	mcz^I	*C. albicans* C CM 110	60	96.7	S
C. glabrata CZ 96a	45.4	95.4	mcz^I	*C. dubliniensis* CM 24	93.1	88.2	S
C. guilliermondii CZ 29	94.4	92.2	itr^I	*C. dubliniensis* CM 31	96.1	95.6	S
C. krusei CM 30b	56.1	95.1	flu^I ket^I mcz^I $5\text{-}FC^I$	*C. dubliniensis* CM 35	70.0	98.1	S
C. krusei CZ 96b	87.0	79.4	flu^I ket^I itr^I mcz^I $5\text{-}FC^I$	*C. dubliniensis* CM 72	60.3	92.5	S
C. krusei CM 20	60.0	94.0	ab^I flu^I ket^I itr^I mcz^I $5\text{-}FC^I$	*C. glabrata* CZ 20	80.0	87.2	S
C. lusitaniae CM 30a	86.2	93.7	$5\text{-}FC^I$	*C. glabrata* CZ 98	54.1	96.3	S

Data was analysed comparing mAbK10 and KP activities of *Candida* isolates with some antifungal resistance versus *Candida* isolates susceptible to the antifungal agents previously tested

[a]mAbK10 and KP were used at a concentration of 20 μg/ml; their activity is expressed as a percentage killing rate evaluated by the cfu assay in comparison with the proper controls

[b]AS, antifungal susceptibility: flu, fluconazole; ke, ketoconazole; itra, itraconazole; mic, miconazole; am, amphotericin B; 5-FC, 5 fluorocytosine; I, intermediately resistant; R, resistant; S, sensitive to all the antifungals tested

Statistical analysis was performed using the Mann-Whitney non parametric test

DPR: DM patients from Parma, Italy; DL: DM patients from London, UK; CM and CZ: non-DM subjects

Table 8.3: mAbK10 and KP candidacidal activity on *Candida* isolates from patients with DM and from non-DM control subjects

D/L *Candida* isolates	K10[a] (%)	KP[a] (%)	D/PR *Candida* isolates	K10[a] (%)	KP[a] (%)	ND *Candida* isolates	K10[a] (%)	KP[a] (%)
C. albicans A DL 2	99	100	*C. albicans* A DPR 59	65	99.7	*C. albicans* A CZ 58	99	92.4
C. albicans A DL 4	97.1	97.7	*C. albicans* A DPR 67	50.1	100	*C. albicans* A CM 75	70.5	76.9
C. albicans A DL 7	77.4	98.6	*C. albicans* A DPR 96	74.3	97.4	*C. albicans* B CM 71	70.2	94.4
C. albicans B DL 67	78.3	61	*C. albicans* A DPR 98	65.9	98.4	*C. albicans* B CM 104	80.2	99.3
C. albicans C DL 54	80.2	90.5	*C. albicans* A DPR 100	90.4	100	*C. albicans* C CM 29	77	90.1
C. dubliniensis DL 63	90.0	91.7	*C. albicans* B DPR 89	76.2	98	*C. dubliniensis* CZ 69	65.2	75.3
C. glabrata DL 48	73.4	96.3	*C. albicans* C DPR74	87.4	97.8	*C. glabrata* CZ 96a	45.4	95.4
C. glabrata DL 102	65.0	83.3	*C. glabrata* DPR 28	99.5	84.4	*C. guilliermondii* CZ 29	94.4	92.2
C. glabrata DL 107	51.6	99.5	*C. glabrata* DPR 33	82.0	96.9	*C. krusei* CM 30b	56.1	95.1
C. guilliermondii DL 73	78.4	95.2	*C. lusitaniae* DPR 31	91.1	98.8	*C. krusei* CZ 96b	87.0	79.4
C. krusei DL 70	45.0	94.2	*C. lusitaniae* DPR 65	56.4	98.7	*C. krusei* CM 20	60.0	94.0
C. parapsilosis DL 98	72.1	93	*C. parapsilosis* DPR 66	30.0	99.3	*C. lusitaniae* CM 30a	86.2	93.7
C. albicans A DL 1	83.4	97.9	*C. albicans* A DPR 38a	52.7	82.9	*C. albicans* A CZ 22	74.4	97.1
C. albicans A DL 18	71	93.1	*C. albicans* A DPR 39	62.4	98	*C. albicans* A CM 76	92.2	93.4
C. albicans A DL 24	70.5	89.8	*C. albicans* A DPR 95	80	97.1	*C. albicans* A CM 95	60	100
C. albicans A DL 66	84.1	92.2	*C. albicans* A DPR 107	97	100	*C. albicans* B CM 23	85.3	84.7
C. albicans A DL 121	78.2	58.2	*C. albicans* B DPR 111	55.3	99	*C. albicans* A CZ 11	81.4	94.4
C. albicans B DL 13	77	92.9	*C. albicans* C DPR 9	91.4	99.8	*C. albicans* C CM 110	60	96.7
C. albicans C DL 84	75	87.2	*C. dubliniensis* DPR 47	73.4	99.0	*C. dubliniensis* CM 24	93.1	88.2
C. dubliniensis DL 41	97.0	89.7	*C. glabrata* DPR 24	62.1	90.5	*C. dubliniensis* CM 31	96.1	95.6
C. dubliniensis DL 92	75.1	92.0	*C. parapsilosis* DPR 23	93.4	94.1	*C. dubliniensis* CM 35	70.0	98.1
C. dubliniensis DL 72	70.0	68.7	*C. parapsilosis* DPR 68	88.0	99.2	*C. dubliniensis* CM 72	60.3	92.5
C. glabrata DL 125	93.0	95.9	*C. tropicalis* DPR 51	50.0	84.0	*C. glabrata* CZ 20	80.0	87.2
C. tropicalis DL 86	78.4	100				*C. glabrata* CZ 98	54.1	96.3

[a]mAbK10 and KP were used at a concentration of 20 μg/ml; their activity is expressed as a percentage killing rate evaluated by the cfu assay in comparison with the proper controls
Statistical analysis was performed using the Kruskal-Wallis non parametric test
DL: DM patients from London, UK; DPR: DM patients from Parma, Italy; ND: non-DM subjects
CM and CZ: non-DM subjects

Table 8.4: KP activity at different concentrations on four selected *Candida* spp. isolates

Candida isolates	AS[a]	KP 20[b] (%)	KP 15[b] (%)	KP 10[b] (%)	KP 5[b] (%)
***C. tropicalis* DL86**	S	100	99.8	98.2	68.4
***C. albicans* A DPR107**	S	100	91.1	47.7	25.5
***C. albicans* A DL2**	itr^R	100	90.4	80	0
***C. glabrata* DL107**	flu^I ket^I itr^I mcz^I	99.5	98.5	95.9	72.8

[a]AS, antifungal susceptibility: S, susceptible to all the antifungals tested; flu, fluconazole; ket, ketoconazole; itr, itraconazole; mcz, miconazole; ab, amphotericin B; 5-FC, 5 fluorocytosine; I, intermediately resistant; R, resistant
[b]KP was tested at 20, 15, 10 and 5 μg/ml; its activity is expressed as a percentage killing rate evaluated by the cfu assay in comparison with the proper control
DL: DM patients from London, UK; DPR: DM patients from Parma, Italy

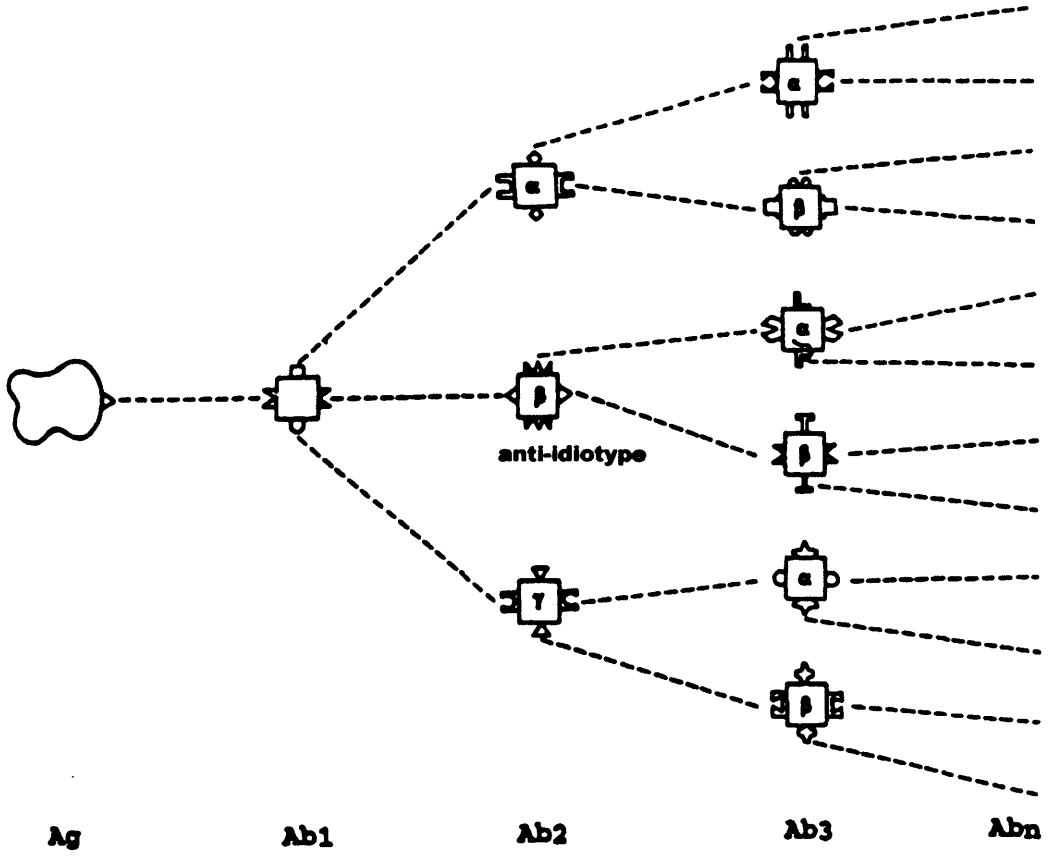

Figure 8.1: A schematic model of the idiotypic cascade

Ab2 β represents the internal image of the Ag epitope

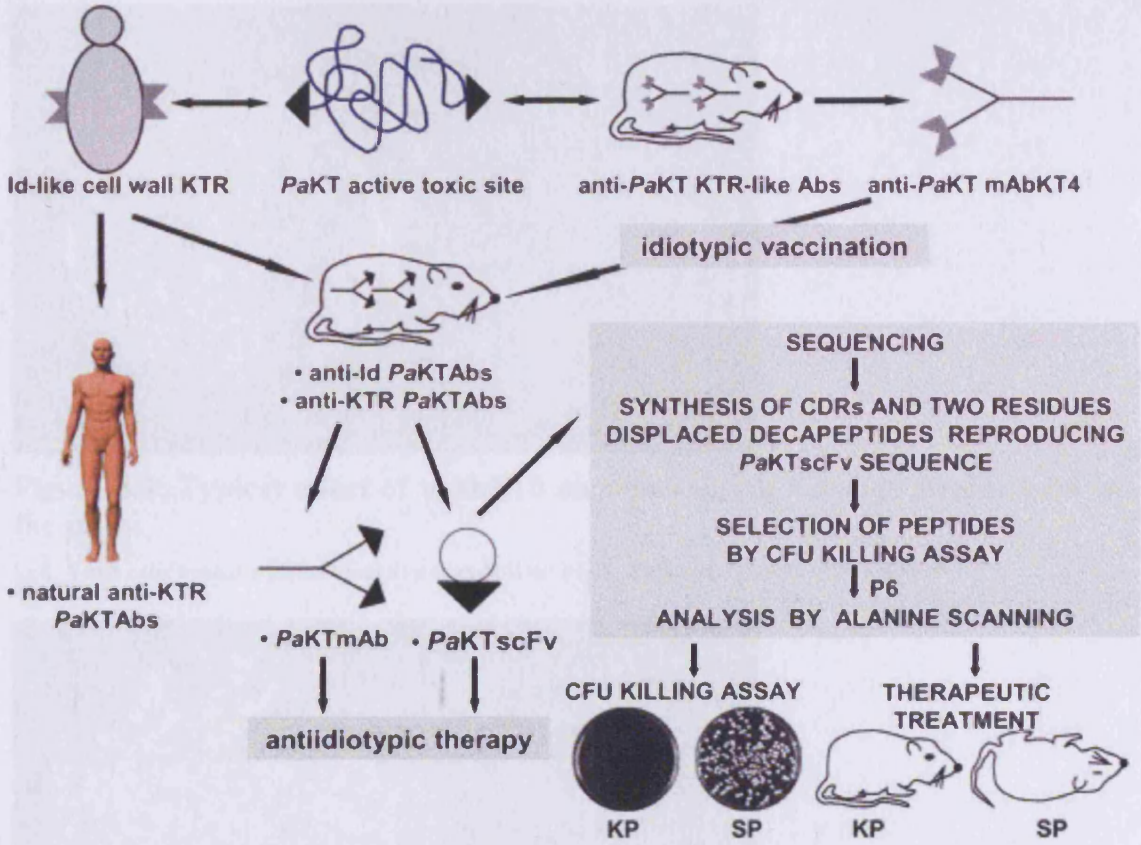

Figure 8.2: Schematic view of KT interactions produced by the yeast *Pichia anomala* (PaKT)

Receptor (KTR)-mediated interactions of *Pichia anomala* killer toxin (PaKT) and PaKT-like natural (anti-KTR PaKTAbs), anti-idiotypic polyclonal (anti-Id PaKTAbs), monoclonal (PaKTmAb), recombinant (PaKTscFv) antibodies and mimotopes (P6, KP) in the idiotypic vaccination and anti-idiotypic therapy (Magliani *et al*, 2004)

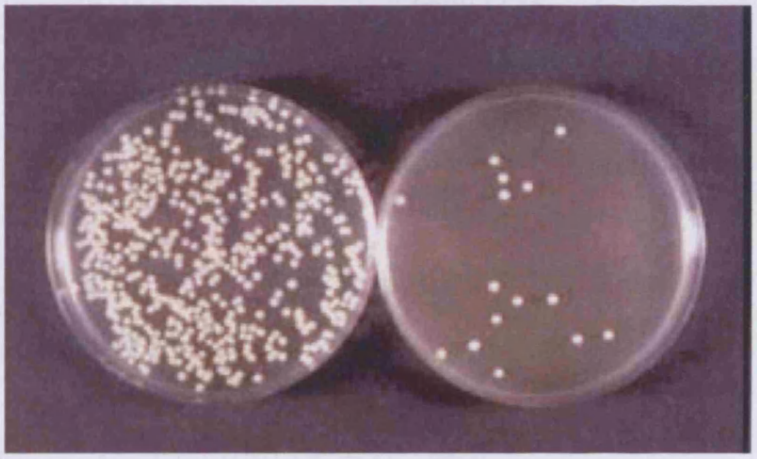

Figure 8.3: Typical effect of mAbK10 on a susceptible *Candida* strains used in the study

Left: Yeast cells treated with heat-inactivated mAbK10; Right: Yeast cells treated with mAbK10

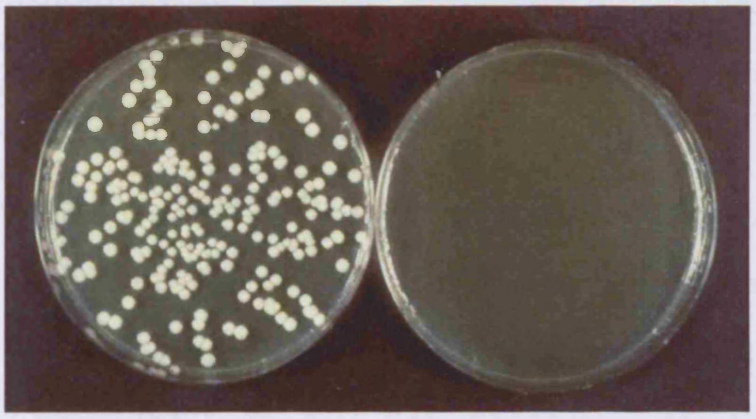

Figure 8.4: Typical effect of the KP on one of the *Candida* strains used in the study

Left: Yeast cells treated with scramble peptide (SP); Right: Yeast cells treated with KP

CHAPTER 9

Summary and conclusions

9.1 Summary

Diabetes mellitus is a common multi-systemic disorder that may result in oral alterations and diseases in affected patients. In particular, oral *Candida* carriage and infections may be more frequent and severe in patients with DM.

This research has investigated the molecular characterisation of oral yeasts isolated from British and Italian patients with DM and from healthy individuals within different dental clinical settings in the United Kingdom.

From this study, a lower than expected incidence (7/249, 2.8%) of oral *Candida* infections among patients affected by DM was observed. This low percentage of oral candidal infection in DM patients seems to contrast with data reported elsewhere (Dorocka-Bobkowska *et al*, 1996; Abu-Elteen & Abu-Alteen, 1998; Willis *et al*, 1999; Guggenheimer *et al*, 2000b; Willis *et al*, 2000b) and cannot be explained solely by clinical awareness or diabetic control. Locality may have influenced the incidence of this disease, although the reasons for this are difficult to ascertain (Manfredi *et al*, 2002). In addition, the results of this investigation on two different groups of DM patients suggest that DM and its parameters of glycaemic control have little or no influence on the oral candidal carriage of patients (Manfredi *et al*, 2002). The overall proportion of DM patients who had *Candida* isolated from their oral cavity in the present study (152/242; 62.8%) was similar to that reported previously (Fisher *et al*, 1987; Aly *et al*, 1992). However, contrary to other published studies where oral yeast colonisation was higher in patients with type 1 DM (Aly *et al*, 1992; Bai *et al*, 1995), there was no significant difference in the present study between the number of type 1

and 2 DM patients whose oral cavity was colonised with yeasts (Rayfield *et al*, 1982; Willis *et al*, 1999). In addition, it would appear that from the comparison of the population of DM patients with a group of healthy non-DM subjects, the former are no more likely to harbour yeast in their oral cavity than healthy control individuals.

There was no notable association between the presence of oral yeasts and genotypic diversity of *C. albicans* of isolates from individuals within different clinical dental settings, and there was no significant difference in the presence of oral yeasts between Italian and UK DM patients. However, a greater ($p=0.04$) number of *C. dubliniensis* isolates were found in the healthy non-DM individuals, and interestingly in the present study both DM patients and control subjects that harboured *C. dubliniensis* were dentate (either fully or partially).

Virulence attributes of *Candida* isolates were studied *in vitro* using adherence and proteinase assays (Section 2.6-2.7). In this study, isolates from patients affected with DM showed a greater ability to adhere ($p<0.0001$) *in vitro* to fibronectin than those isolated from healthy subjects. This observation could indicate that a higher concentration of glucose in the saliva of DM patients may enhance the ability of *Candida* to adhere *in vitro*, as reported previously (Odds *et al*, 1978; Samaranayake *et al*, 1984b). Although no differences were observed between isolates from DM and non-DM subjects in the *in vitro* production of extracellular proteinase, oral yeast isolates from patients with type 2 DM produced more extracellular proteinase ($p=0.02$) than those from patients with type 1 DM. The difference in the expression of this virulence attribute may reflect variations of certain constituents in saliva of type 2 DM patients, such as higher salivary IgA levels and certain salivary enzymes *e.g.* metalloproteinases (Collin *et al*, 2000b).

In the present study, an intriguing relationship between lower candidal load (<100 cfu/ml) and a higher *in vitro* ability to adhere was observed for all the *Candida*

carriers evaluated. The assay used to assess *in vitro* adhesion directly measured the ability of isolates to adhere to fibronectin coated with dynabeads (Dynal, UK). Therefore, it can be assumed that, as the number of yeasts present in the oral cavity increases, interactions between yeasts become more important than those between the yeast and the host, as was found in this study. Such a theory supports the concept of biofilm formation (Baillie & Douglas, 1998) where interactions between microorganisms appear to be of greater significance than those with the host surfaces themselves (Sullivan & Coleman, 1997; Willis *et al*, 2000a).

The most notable observation of this research study was that oral prostheses may influence not only the oral carriage and the species of oral *Candida* but also the capability of oral yeasts to adhere in DM patients. Dentures may function as a chronic reservoir of yeasts inoculation and dissemination (Davenport, 1970; Verran & Maryan, 1997; Maza *et al*, 2002), and surface irregularities would increase the likelihood of microorganisms remaining on the surface after the prosthesis has been cleaned. Increased oral candidal load is much more common in full-denture wearers than in dentate individuals (Parvinen *et al*, 1994; Manfredi *et al*, 2002). It may also be the case that DM, rather than the presence of a dental prosthesis, affects adherence, as the ability of isolates from the control subgroup to adhere was not influenced by the presence of dentures. In addition, any enhanced interactions between yeasts and local factors such as a low salivary medium (e.g. due to the presence of dentures) or poor metabolic control may increase the ability of *Candida* to adhere to oral mucosa, rather than any yeast-derived factor.

As well as dentures, locality may influence different aspects of oral yeasts. *In vitro* antifungal susceptibility testing established that isolates from UK DM patients were much more resistant to azole antifungal agents than those from Italian patients. The difference in the antifungal resistance of isolates from the two populations of DM

patients may reflect differences in the therapeutic management of candidal infections between the two centres in Italy and UK; however, there was no long-term data available to support this notion. Although no association could be observed in the DM status of the patients in London and Parma, it could be assumed that as the London group had more long-standing DM patients with more complications, they would be more likely to have received or taken the antifungal agents themselves. In this study, *C. albicans* strains were more susceptible to fluconazole and miconazole than non-*C. albicans* strains when evaluated using a commercial kit, Fungitest (Bio-Rad). This could be considered a useful kit for *in vitro* evaluation of fluconazole sensitivity among *Candida* spp. isolates in clinical laboratories, with positive agreement among laboratories regarding its reproducibility, according to recent reports (Morace *et al*, 2002). The results obtained in the present study confirm that previously reported emergence of *C. albicans* fluconazole and, more generally, triazole resistance was probably due to the non-standardised susceptibility methods rather than a real shift towards more resistant *Candida* species.

It has been reported that *C. albicans* genotype B and C (both characterised by the presence of Group I intron in the 25S rDNA) showed a greater susceptibility to 5-FC than intronless strain *C. albicans* genotype A (Mercure *et al*, 1993; McCullough *et al*, 1999b). In this study, no statistical differences were observed in the 5-FC susceptibility of *C. albicans* genotype B isolates from all the carriers evaluated and only one isolate from subgroup C (Appendix 2, C/M 29) expressed an intermediate resistance to this antifungal agent. Probably the contribution of the Group I intron of the 25S rDNA to the susceptibility of *C. albicans* genotypes to the 5-FC susceptibility is not the only one factor implicated in the complex process of yeast antifungal resistance.

PCR fingerprinting, with subsequent phylogenetic analysis on *C. albicans* isolates from all subjects, revealed that there were significant differences between *C. albicans* isolates, indicative of the distinct ecological niches that occur in the oral cavities of these patient cohorts. The most diverse group was isolates from the DM patients in the UK, probably reflecting the antifungal treatment that these patients have received. Finally, the activity of a rat IgM monoclonal anti-idiotypic antibody (mAbK10) and a synthetic decapeptide (KP) derived from the sequence of a single-chain, recombinant, anti-idiotypic antibody, was evaluated for the first time on different selected *Candida* spp. isolates from the two DM populations and from the non-DM control group. In the present study, both mAbK10 and KP were found to be extremely active against wide spectra of oral isolates of different *Candida* spp., and both *C. albicans* and non-*C. albicans* spp. were susceptible to mAbK10 and KP. Likewise, strains resistant to conventional antifungal agents were also susceptible to the antifungal activity of these two new agents, suggesting new perspectives in the design and production of candidacidal compounds whose mechanism reflects that exerted in nature by killer yeasts.

9.2 Conclusions

The evaluation of the molecular characteristics of *Candida* isolates from two geographically different groups of DM patients and from healthy non-DM control subjects has led to the conclusion that local factors, such as the presence of dental prostheses, associated with DM, may influence the carriage, species and virulence attributes of oral *Candida* yeasts more than systemic factors alone.

Geographical locality may also influence different properties of *Candida* spp., but further studies including the study of patients from more diverse geographic locales are necessary to establish this association. In particular, the results obtained from the

study of a group of non-DM healthy subjects enrolled in Italy should be analysed together with those evaluated in this thesis to better establish the correlations reported.

Research could include the evaluation of the expression of different virulence genes (*e.g.* SAP and ALS) using RT-PCR for the *Candida* isolates grown in the same media used for the adhesion and proteinase activity assays in the present study.

Interesting differences were observed from the PCR fingerprinting analysis of the *C. albicans* isolates of DM and non-DM subjects, indicative of the occurrence of distinct ecological niches in the oral cavities of these patient cohorts. It may be of value to extend the analysis to a larger grcup of *C. albicans* isolates of patients with DM and without DM from different geographic localities in order to evaluate *C. albicans* genetic relatedness in a particular group of carriers.

The significant *in vitro* candidacidal activity of both K10 and KP on the *Candida* isolates evaluated in this study suggests that the activity of the two molecules could be further evaluated in an *in vivo* experimental model of animal oral candidosis, in order to confirm (or not) the results already obtained in a previous study (Polonelli *et al*, 2003) which successfully reported KP activity on a mice vaginal candidosis model.

CHAPTER 10

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Appendix 1: Diagnostic Criteria for DM

(The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998)

Diabetes mellitus may be diagnosed by any one of 3 methods. Whatever method is used it must be repeated on a subsequent day.

1. Symptoms of diabetes plus random (not fasting) plasma glucose ≥200 mg/dl. The blood for examination of glucose may be drawn at any time of day and without regard to time since the last meal. The relevant symptoms of diabetes include polyuria, polydypsia and unexplained weight loss.

or

2. Fasting plasma glucose ≥126 mg/dl. Fasting is defined as no caloric intake for at least 8 h.

or

3. Two-hour post-prandial glucose ≥200 mg/dl during an oral glucose tolerance test (OGTT). The test should be performed using a glucose load containing the equivalent of 75 g of anhydrous glucose dissolved in water. This method is not recommended for routine clinical use

Categories of 2-hour post-prandial glucose (2hPG) include:

1. 2hPG< 140 mg/dl = normal glucose tolerance
2. 2hPG ≥ 140 mg/dl and <200 mg/dl = impaired glucose tolerance
3. 2hPG ≥200 mg/dl = provisional diagnosis of diabetes (this must be confirmed on subsequent day)

Categories of fasting plasma glucose (FPG) include:

FPG<110 mg/dl =normal fasting glucose

FPG ≥ 110 mg/dl and <126 mg/dl = Impaired fasting glucose

FPG ≥ 126 mg/dl = provisional diagnosis of diabetes (this must be confirmed on subsequent day)

Appendix 2: *Candida* isolates evaluated in the study with the antifungal susceptibilities of each isolate

Candida isolate	Species	F	M	I	K	AB	5-FC
D/L1	*C. albicans* A	S	S	S	S	S	S
D/L2	*C. albicans* A	S	S	R	S	S	S
D/L4	*C. albicans* A	S	IR	S	S	S	S
D/L6	*C. albicans* A	S	IR	S	S	S	S
D/L7	*C. albicans* A	S	S	R	R	S	R
D/L8	*C. albicans* A	S	S	S	R	S	S
D/L9	*C. albicans* A	S	S	IR	S	S	S
D/L10	*C. albicans* A	S	S	S	R	S	S
D/L11	*C. albicans* A	S	S	S	S	S	S
D/L13	*C. albicans* B	S	S	S	S	S	S
D/L15	*C. albicans* A	S	S	S	S	S	S
D/L16	*C. albicans* A	S	S	IR	IR	S	S
D/L17	*C. albicans* A	S	S	R	R	S	S
D/L18	*C. albicans* A	S	S	S	S	S	S
D/L19	*C. albicans* A	S	S	S	R	S	S
D/L20	*C. albicans* A	S	S	S	S	S	S
D/L21	*C. albicans* A	S	S	S	S	S	S
D/L24	*C. albicans* A	S	S	S	S	S	S
D/L25	*C. albicans* A	S	S	S	S	S	S
D/L26	*C. albicans* A	S	S	R	R	S	S
D/L27	*C. albicans* A	S	S	R	R	IR	IR
D/L30	*C. albicans* A	IR	IR	IR	IR	IR	S
D/L31	*C. albicans* A	S	S	S	S	S	S
D/L32	*C. albicans* A	S	S	S	S	S	S
D/L34	*C. albicans* A	IR	IR	IR	IR	S	S
D/L35	*C. albicans* A	S	S	S	S	S	S
D/L36	*C. albicans* A	S	S	S	S	S	S
D/L39	*C. albicans* A	S	IR	S	S	S	S
D/L40	*C. albicans* A	S	S	S	S	S	S
D/L41	*C. dubliniensis*	S	S	S	S	S	S
D/L42	*C. albicans* B	S	S	S	S	S	S
D/L43	*C. albicans* A	S	IR	S	S	IR	S
D/L44	*C. albicans* B	S	S	S	S	IR	S
D/L46	*C. albicans* A	IR	IR	IR	IR	S	S
D/L48	*C. glabrata*	IR	IR	IR	S	IR	S
D/L50	*C. albicans* A	S	IR	IR	S	S	S
D/L54	*C. albicans* C	S	IR	S	S	S	S
D/L61	*C. albicans* A	S	IR	S	S	S	S
D/L62	*C. albicans* A	IR	IR	S	S	IR	IR
D/L63	*C. dubliniensis*	IR	IR	S	S	S	S
D/L64	*C. albicans* A	IR	IR	R	IR	S	S
D/L65	*C. dubliniensis*	S	IR	S	S	S	S
D/L66	*C. albicans* A	S	S	S	S	S	S

Candida isolate	Species	F	M	I	K	AB	5-FC
D/L67	*C. albicans* B	R	IR	R	IR	S	S
D/L69	*C. albicans* A	S	IR	S	S	S	S
D/L70	*C. krusei*	IR	IR	R	S	S	S
D/L72	*C. dubliniensis*	S	S	S	S	S	S
D/L73	*C. guilliermondii*	S	IR	IR	IR	S	S
D/L77	*C. albicans* B	S	IR	S	S	S	S
D/L78	*C. albicans* A	S	S	S	S	S	S
D/L81	*C. albicans* C	S	IR	S	S	S	S
D/L84	*C. albicans* C	S	S	S	S	S	S
D/L86	*C. tropicalis*	S	S	S	S	S	S
D/L87	*C. albicans* A	S	S	S	S	S	S
D/L91	*C. albicans* A	R	IR	IR	IR	S	IR
D/L92	*C. dubliniensis*	S	S	S	S	S	S
D/L94	*C. albicans* A	IR	IR	R	R	S	S
D/L95	*C. albicans* A	S	S	S	S	S	S
D/L96	*C. albicans* A	S	S	S	S	S	S
D/L98	*C. parapsilosis*	IR	IR	S	S	S	IR
D/L100	*C. albicans* A	IR	S	IR	IR	S	S
D/L101	*C. albicans* A	S	S	S	S	S	S
D/L102	*C. glabrata*	R	IR	R	R	S	S
D/L103	*C. albicans* A	S	S	S	S	S	S
D/L107	*C. glabrata*	IR	IR	IR	IR	S	S
D/L108	*C. albicans* A	S	S	S	S	S	S
D/L109	*C. glabrata*	S	S	S	IR	S	S
D/L110	*C. albicans* A	S	S	S	S	S	S
D/L111	*C. albicans* A	R	IR	R	IR	S	S
D/L112	*C. albicans* A	R	IR	R	R	S	S
D/L113	*C. albicans* A	S	S	S	S	S	S
D/L114	*C. albicans* A	S	S	S	S	S	S
D/L115	*C. albicans* A	S	S	S	S	S	S
D/L116	*C. albicans* A	S	S	S	S	S	S
D/L118	*C. albicans* B	S	S	S	S	S	S
D/L121	*C. albicans* A	S	S	S	S	S	S
D/L122	*C. albicans* A	S	S	S	S	S	S
D/L124	*C. albicans* A	S	S	S	S	S	S
D/L125	*C. glabrata*	S	S	S	S	S	S
D/L126	*C. albicans* A	S	S	S	S	S	S
D/L135	*C. albicans* A	S	S	S	S	S	S
D/L137	*C. albicans* B	S	S	S	S	S	S
D/L139	*C. albicans* A	S	S	S	R	S	S
D/PR1	*C. albicans* A	S	S	S	S	S	S
D/PR2	*C. albicans* A	S	S	S	S	S	S
D/PR3	*C. albicans* A	S	S	S	S	S	S
D/PR4	*C. albicans* A	S	S	S	S	S	S
D/PR6	*C. glabrata*	S	S	S	S	S	S
D/PR8	*C. albicans* A	S	S	S	S	S	S

Candida isolate	Species	F	M	I	K	AB	5-FC
D/PR9	*C. albicans* C	S	S	S	S	S	S
D/PR12	*C. albicans* A	S	S	S	S	S	S
D/PR15	*C. albicans* A	S	S	S	S	S	S
D/PR17	*C. albicans* A	S	S	S	S	S	S
D/PR18	*C. albicans* A	S	S	S	S	S	S
D/PR23	*C. parapsilosis*	S	S	S	S	S	S
D/PR24	*C. glabrata*	S	S	S	S	S	S
D/PR25	*C. albicans* A	S	S	S	S	S	S
D/PR27	*C. albicans* A	S	S	S	S	S	S
D/PR28	*C. glabrata*	IR	IR	IR	S	S	S
D/PR30	*C. albicans* A	S	S	S	S	S	S
D/PR31	*C. lusitaniae*	IR	IR	IR	IR	S	IR
D/PR32	*C. albicans* A	S	S	S	S	S	S
D/PR33	*C. glabrata*	S	S	IR	S	S	S
D/PR34	*C. albicans* A	S	S	S	S	S	S
D/PR35	*C. albicans* A	S	S	S	S	S	S
D/PR38a	*C. albicans* A	S	S	S	S	S	S
D/PR38b	*C. albicans* B	S	S	S	S	S	S
D/PR39	*C. albicans* A	S	S	S	S	S	S
D/PR40	*C. albicans* B	S	S	S	S	S	S
D/PR41	*C. albicans* B	S	S	S	S	S	S
D/PR44	*C. albicans* B	S	S	S	S	S	S
D/PR45	*C. albicans* A	S	S	S	S	S	S
D/PR46	*C. albicans* A	S	S	S	S	S	S
D/PR47	*C. dubliniensis*	S	S	S	S	S	S
D/PR51	*C. tropicalis*	S	S	S	S	S	S
D/PR54	*C. parapsilosis*	S	S	S	S	S	S
D/PR55	*C. glabrata*	S	S	S	S	S	S
D/PR56	*C. albicans* A	S	S	S	S	S	S
D/PR57	*C. albicans* A	S	S	S	S	S	S
D/PR58	*C. albicans* A	S	S	S	S	S	S
D/PR59	*C. albicans* A	S	S	IR	S	S	S
D/PR65	*C. lusitaniae*	IR	IR	IR	IR	S	IR
D/PR66	*C. parapsilosis*	S	S	S	IR	S	S
D/PR67	*C. albicans* A	S	S	S	S	S	R
D/PR68	*C. parapsilosis*	S	S	S	S	S	S
D/PR71	*C. albicans* A	S	S	S	S	S	S
D/PR72a	*C. albicans* B	S	S	S	S	S	S
D/PR72b	*C. glabrata*	S	S	S	S	S	S
D/PR73	*C. albicans* A	S	S	S	S	S	S
D/PR74	*C. albicans* C	S	S	IR	S	S	S
D/PR75	*C. albicans* A	S	S	S	S	S	S
D/PR76	*C. albicans* A	S	S	S	S	S	S
D/PR79	*C. albicans* A	S	S	S	S	S	S
D/PR81	*C. parapsilosis*	S	S	S	S	S	S
D/PR82	*C. glabrata*	S	S	S	S	S	S

Candida isolate	Species	F	M	I	K	AB	5-FC
D/PR84	*C. albicans* B	S	S	S	S	S	S
D/PR86	*C. albicans* A	S	S	S	S	S	S
D/PR89	*C. albicans* B	S	S	S	IR	S	S
D/PR90	*C. albicans* A	S	S	S	S	S	S
D/PR93	*C. albicans* B	S	S	S	S	S	S
D/PR94	*C. albicans* A	S	S	S	S	S	S
D/PR95	*C. albicans* A	S	S	S	S	S	S
D/PR96	*C. albicans* A	S	S	IR	S	S	S
D/PR97	*C. albicans* A	S	S	S	S	S	S
D/PR98	*C. albicans* A	R	S	R	IR	S	S
D/PR100	*C. albicans* A	S	S	IR	S	S	S
D/PR101	*C. parapsilosis*	S	S	S	S	S	S
D/PR102	*C. parapsilosis*	S	S	S	S	S	S
D/PR105	*C. albicans* B	S	S	R	IR	S	S
D/PR106	*C. tropicalis*	IR	IR	IR	IR	IR	S
D/PR107	*C. albicans* A	S	S	S	S	S	S
D/PR109	*C. albicans* A	S	S	S	IR	S	S
D/PR110	*C. albicans* A	S	S	S	S	S	S
D/PR111	*C. albicans* B	S	S	S	S	S	S
C/Z 24	*C. albicans* A	S	S	S	S	S	S
C/Z 16	*C. albicans* A	S	S	S	S	S	S
C/Z 15	*C. albicans* A	S	S	S	S	S	S
C/Z 21	*C. albicans* A	S	S	S	S	S	S
C/Z 28	*C. albicans* B	S	IR	S	S	S	S
C/Z 22	*C. albicans* A	S	S	S	S	S	S
C/Z 38	*C. albicans* A	S	S	S	S	S	S
C/Z 36	*C. albicans* A	IR	S	S	S	S	S
C/Z 44	*C. albicans* B	S	S	S	S	S	S
C/Z 43	*C. albicans* A	S	S	S	S	S	S
C/Z 37	*C. albicans* A	S	S	S	S	S	S
C/Z 57	*C. albicans* A	S	S	S	S	S	S
C/Z 105	*C. albicans* A	S	S	S	S	S	S
C/Z 102	*C. albicans* A	S	S	S	S	S	S
C/Z 104	*C. albicans* A	S	IR	S	S	IR	S
C/Z 19	*C. glabrata*	IR	S	IR	S	S	S
C/Z 41	*C. albicans* A	S	S	S	S	S	S
C/Z 49	*C. glabrata*	IR	IR	IR	IR	S	S
C/Z 50	*C. albicans* A	S	S	S	R	S	S
C/Z 61	*C. albicans* B	S	S	S	S	S	S
C/Z 63	*C. albicans* B	S	S	S	S	IR	S
C/Z 98	*C. glabrata*	S	S	S	S	S	S
C/M 96	*C. albicans* A	S	S	S	S	S	S
C/Z 100	*C. albicans* A	S	S	S	S	S	S
C/Z 69	*C. dubliniensis*	S	IR	S	S	S	S
C/Z 20	*C. glabrata*	S	S	S	S	S	S
C/M 55	*C. albicans* A	S	S	S	S	S	IR

Candida isolate	Species	F	M	I	K	AB	5-FC
C/Z 17	*C. albicans* A	S	S	S	S	S	S
C/M 104	*C. albicans* B	S	IR	S	S	S	S
C/Z 90	*C. albicans* A	S	IR	S	S	S	S
C/Z 96a	*C. glabrata*	S	IR	S	S	S	S
C/Z 96b	*C. krusei*	IR	IR	IR	IR	S	IR
C/Z 99	*C. albicans* A	S	IR	S	S	S	S
C/Z 97	*C. albicans* A	S	IR	S	S	S	S
C/Z 103	*C. albicans* A	S	IR	S	S	S	S
C/M 18	*C. albicans* A	S	S	S	S	IR	S
C/M 19	*C. albicans* A	S	S	S	S	IR	S
C/M 20	*C. krusei*	IR	IR	IR	IR	IR	IR
C/M 21	*C. albicans* A	IR	S	IR	S	IR	S
C/M 23	*C. albicans* B	S	S	S	S	S	S
C/M 24	*C. dubliniensis*	S	S	S	S	S	S
C/M 29	*C. albicans* C	S	S	S	S	S	IR
C/M 30a	*C. lusitaniae*	S	S	S	S	S	IR
C/M 30b	*C. krusei*	IR	IR	S	IR	S	IR
C/M 31	*C. dubliniensis*	S	S	S	S	S	S
C/M 71	*C. albicans* B	S	S	R	IR	S	S
C/M 72	*C. dubliniensis*	S	S	S	S	S	S
C/M 73	*C. dubliniensis*	S	S	S	S	S	S
C/M 74	*C. dubliniensis*	S	S	S	S	S	S
C/M75	*C. albicans* A	R	IR	R	R	IR	S
C/M 76	*C. albicans* A	S	S	S	S	S	S
C/M 77	*C. albicans* A	S	S	S	S	S	S
C/M 78	*C. dubliniensis*	S	S	S	S	S	S
C/M 80	*C. dubliniensis*	S	S	S	S	S	S
C/M 81	*C. albicans* A	S	S	IR	S	S	S
C/M 84	*C. albicans* B	S	S	S	S	S	S
C/M 85	*C. albicans* A	S	S	S	S	S	S
C/M 94	*C. albicans* A	S	S	R	R	S	S
C/M 95	*C. albicans* A	S	S	S	S	S	S
C/Z 11	*C. albicans* A	S	S	S	S	S	S
C/Z 13	*C. albicans A*	S	S	S	S	S	S
C/Z 29	*C. guilliermondii*	S	S	IR	S	S	S
C/Z 48	*C. albicans* A	S	S	S	S	S	S
C/Z 54	*C. albicans* A	S	S	S	S	S	S
C/Z 58	*C. albicans* A	S	S	R	S	S	S
C/Z 56	*C. albicans* A	S	S	S	S	S	S
C/Z 32	*C. albicans* A	IR	IR	S	S	S	IR
C/Z 78	*C. albicans* A	S	IR	S	S	S	S
C/Z 25	*C. albicans* A	S	S	S	S	S	S
C/M 88	*C. albicans* A	S	S	S	S	S	S
C/Z 30	*C. albicans* A	S	S	S	S	S	S
C/M 110	*C. albicans* C	S	S	S	S	S	S
C/M 46	*C. albicans* A	S	S	S	S	IR	IR

Candida isolate	Species	F	M	I	K	AB	5-FC
C/M 35	*C. dubliniensis*	S	S	S	S	S	S
C/M 27	*C. albicans* A	S	S	S	S	S	S

D/L: DM from London, UK; D/PR: DM from Parma, Italy; C/M and C/Z non-DM subjects
F: fluconazole; M: miconazole; I: itraconazole; K: ketoconazole; AB: amphotericine B; 5-FC: 5-fluorocytosine
S: Susceptible; IR: intermediately resistant; R: resistant

Attachments

TO WHOM IT MAY CONCERN

The Harvard method of referencing has been used in the thesis as agreed with UCL.

London, 06/02/2006

Oral Microbiology Immunology 2002: 17: 44–49
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Oral Microbiology and Immunology
ISSN 0902-0055

Molecular characterization of *Candida* spp. isolated from the oral cavities of patients from diverse clinical settings

Al-Karaawi Z M, Manfredi M, Waugh A C W, McCullough M J, Jorge J, Scully C, Porter S R. Molecular characterization of Candida *spp. isolated from the oral cavities of patients from diverse clinical settings.*
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Oral Microbiology and Immunology
ISSN 0902-0055

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Oral Diseases (2004) 10, 187–200

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REVIEW ARTICLE

Update on diabetes mellitus and related oral diseases

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Oral Microbiology Immunology 2005: 20: 226–232
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